PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	**FOR FURTHER ACTION** see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
MJL/1092.2/C		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/GB 00/02962	31/07/2000	29/07/1999
Applicant		
CYTOCELL LIMITED		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [X] contained in the international application in written form.

   [X] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [ ] the text is approved as submitted by the applicant.

   [X] the text has been established by this Authority to read as follows:

   METHOD FOR DETECTING NUCLEIC ACID TARGET SEQUENCES INVOLVING IN VITRO TRANSCRIPTION FROM AN RNA POLYMERASE PROMOTER

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 1

   [X] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PC[redacted] 00/02962

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	WO 99 37805 A (CARDY DONALD LEONARD NICHOLAS ;RAY TREVOR DUNCAN (GB); MARSH PETER) 29 July 1999 (1999-07-29) page 6, paragraph 6; figures 1,2 page 9, paragraph 2 -page 10, paragraph 3 page 7, paragraph 2 -page 8, paragraph 2 ---	1-17
P,Y	WO 99 37806 A (CARDY DONALD LEONARD NICHOLAS ;RAY TREVOR DUNCAN (GB); MARSH PETER) 29 July 1999 (1999-07-29) page 4, paragraph 2; figures 1,9,11A,11B,13A,14A; examples 6,9 ---	1-17
A	EP 0 851 033 A (GEN PROBE INC) 1 July 1998 (1998-07-01) page 10, line 31 -page 13, line 3 ---	1

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 November 2000	28/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Osborne, H

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PC[redacted] 00/02962

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93 06240 A (CYTOCELL LTD) 1 April 1993 (1993-04-01) figure 2	1
A	EP 0 552 931 A (GEN PROBE INC) 28 July 1993 (1993-07-28) figure 15C	1

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PC[redacted]B 00/02962

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9937805	A	29-07-1999	AU	2431799 A	09-08-1999
			EP	1049806 A	08-11-2000
WO 9937806	A	29-07-1999	AU	2289299 A	09-08-1999
			EP	1051515 A	15-11-2000
EP 0851033	A	01-07-1998	US	6025133 A	15-02-2000
			AU	5525998 A	31-07-1998
			WO	9829568 A	09-07-1998
WO 9306240	A	01-04-1993	AT	152778 T	15-05-1997
			AU	672367 B	03-10-1996
			AU	2558692 A	27-04-1993
			CA	2118913 A	01-04-1993
			DE	69219627 D	12-06-1997
			DE	69219627 T	04-09-1997
			DK	666927 T	15-09-1997
			EP	0666927 A	16-08-1995
			ES	2101116 T	01-07-1997
			JP	6510669 T	01-12-1994
EP 0552931	A	28-07-1993	AU	665062 B	14-12-1995
			AU	3586693 A	01-09-1993
			CA	2128530 A	05-08-1993
			DE	69328699 D	29-06-2000
			DE	69328699 T	07-09-2000
			JP	7503139 T	06-04-1995
			WO	9315102 A	05-08-1993
			US	5451503 A	19-09-1995
			US	5424413 A	13-06-1995

10/pn

PCT/GB00/02962

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 11 April 2001 (11.04.01)	
International application No. PCT/GB00/02962	**Applicant's or agent's file reference** MJL/1092.2/C
International filing date (day/month/year) 31 July 2000 (31.07.00)	**Priority date** (day/month/year) 29 July 1999 (29.07.99)
Applicant LLOYD, John, Scott et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   09 January 2001 (09.01.01)

   [ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Juan Cruz
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

GB0002962

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
MJL/1092.2/C		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB00/02962	31/07/2000	29/07/1999
International Patent Classification (IPC) or national classification and IPC C12Q1/68		
Applicant CYTOCELL LIMITED et al		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 8 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☒ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
09/01/2001	04.10.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Knudsen, H Telephone No. +49 89 2399 8696

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB00/02962

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-29	as received on	31/08/2000	with letter of	24/08/2000

Claims, No.:

1-18	as received on	04/08/2001	with letter of	10/07/2001

Drawings, sheets:

1/10-10/10	as received on	31/08/2000	with letter of	24/08/2000

Sequence listing part of the description, pages:

1-11, as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   ☐ the language of publication of the international application (under Rule 48.3(b)).
   ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   ☒ contained in the international application in written form.
   ☒ filed together with the international application in computer readable form.
   ☐ furnished subsequently to this Authority in written form.
   ☐ furnished subsequently to this Authority in computer readable form.
   ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

☐ the description, pages:

☐ the claims, Nos.:

☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

☐ copy of the earlier application whose priority has been claimed.

☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 16-17.

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the said claims Nos. 16-17.

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

☐ restricted the claims.

☐ paid additional fees.

☐ paid additional fees under protest.

☐ neither restricted nor paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is

☐ complied with.

☒ not complied with for the following reasons:
see separate sheet

4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:

☒ all parts.

☐ the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims 1-15,18
	No:	Claims

Inventive step (IS)	Yes:	Claims	1-15,18
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-15,18
	No:	Claims	

2. Citations and explanations
see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item II
Priority

The priority is validly claimed for present claims 1-15 and 18 and the P-documents mentioned in the International Search Report therefore are not taken into consideration in the present International examination of these claims.

Re Item III
Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The kits of present claims 16-17 go beyond what has been searched in the International Search. The subject-matter of original claims 16-17 were a different complex and method employing only two probes which were completely different from the probes mentioned in the present claims. The subject-matter of original claims 1-15 was directed to complexes or methods in which three different probes formed a complex with a target. Thus, kits containing only two of the three probes were not subject of a search and therefore cannot be examined. No examination is therefore carried out for claims 16-17.

Re Item V
Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

CITED PRIOR ART DOCUMENTS:

5.1 EP-A-0 851 033 (D3) discloses the use of a single stem-loop probe containing a ss RNA polymerase promoter region. A further probe, which is complementary to the promoter region, is used to create a ds RNA polymerase promoter region. RNA produced by a RNA polymerase is detected and indicates whether binding has taken place.

5.2 WO 93/06240 (D1) discloses a method in which two probes which both bind a target sequence and if bonded to the target sequence binds to each other. One of the probes may be extended by a DNA polymerase to create a RNA polymerase promoter which can be used for producing RNA indicative of binding to the target.

5.3 EP-A-0 552 931 (D2) discloses a similar assay to that of D1 (see Fig.15). Moreover, D2 discloses an assay in which the complementary region of the probes (the arm) contain a RNA polymerase promoter (see claim 31). The RNA produced is detected.

NOVELTY:

5.4 None of D1-D3 disclose the formation of a RNA polymerase promoter in which two probes form one strand of the promoter. Claims 1-15 and 18 are therefore novel.

INVENTIVE STEP:

5.5 None of D1-D3 suggest the possibility of creating a RNA polymerase promoter by using two different probes for forming one strand thereof and claims 1-15 are therefore not considered obvious to the skilled person.

INDUSTRIAL APPLICABILITY:

5.6 Claims 1-15 and 18 concern complexes. kits and in-vitro assays and are therefore considered industrially applicable.

Re Item VI

Certain documents cited

Certain published documents (Rule 70.10)

Application No Patent No	Publication date *(day/month/year)*	Filing date *(day/month/year)*	Priority date *(valid claim)* *(day/month/year)*
WO 99 37806	26.01.1999	27.01.1998	29.04.1998
WO 99/37805	29.07.1999	26.01.1999	27.01.1998 08.07.1998

The above documents are both published on the present application's priority date, and before its filing date. They are therefore relevant for only those parts of the present application, if any, which do not have a valid claim to priority. Moreover, the above documents may become relevant prior art in the Regional phase of the present application.

Re Item VII

Certain defects in the international application

7.1 Contrary to the requirements of Rule 5(a)(ii) PCT, the closest prior art document D1 is not identified in the description and the relevant background art disclosed therein is not briefly discussed.

7.2 It is not possible to incorporate the teaching of a prior art document into the present application's disclosure by the expression "herein incorporated by reference" or equivalents thereof (see p.2, third paragraph) (PCT Guidelines, C-II, 4.17).

Re Item VIII

Certain observations on the international application

8.1 The expression "substantially adjacent" used in claims 1 and 12 is vague. The definition in the description seems to have sufficient clarity.

8.2 One major technical problem when using complementary probes in assays is to avoid the probes hybridizing in the absence of target. However, present claim 12 does not contain any features which are directed to avoiding this problem. The applicant notes that Example 1 shows that the method is feasible without a destabilising agent (ie the feature of claim 9), but does not explain how this technical problem is solved.

8.3 The use of a discontinuous promoter formed by the second and third promoter seems to be contradictory to the statement in the description (p.4, l.16-18) (see also # 8.1). The object of claims 10-11 is therefore not clear. The applicant argues that it is one strand of the promoter in which the probes are not covalently bound which is considered discontinuous, but this would still appear to be contrary the object of the invention as defined in the said statement.

8.4 The complex in present claims 1-11 is an intermediate in the assay of claim 12. The intermediate is neither used as such nor isolated at any stage of the claimed assay. It would therefore seem logical to claim the assay and optionally a kit containing the probes instead of the complex. The applicant is invited to comment on this remark.

Claims

1. A complex formed by a hybridisation reaction comprising four nucleic acid molecules; the complex comprising a target nucleic acid molecule and first, second and third nucleic acid probe molecules; wherein the first probe comprises a foot region which is complementary to a first portion of the target and is hybridised thereto, and an arm region which is substantially non-complementary to the target; the second probe comprises a foot region which is complementary to a second portion of the target, such that the foot region of the second probe is hybridised to the target adjacent or substantially adjacent to the foot region of the first probe, the second probe also comprising an arm region which is substantially non-complementary to the target but which is complementary and hybridised to the arm region of the first probe; the third probe being complementary, at least in part, to a portion of the arm region of the first probe, such that the third probe is hybridised to the arm region of the first probe adjacent or substantially adjacent to the second probe; and wherein formation of the complex creates a functional double-stranded RNA polymerase promoter, one strand of the promoter being provided by the first probe, and the other strand being provided jointly by the second probe and by the third probe.

2. A complex according to claim 1, wherein at least one of the first, second or third probes comprises PNA and/or LNA.

3. A complex according to claim 2, wherein the first and/or second probe comprises PNA and/or LNA.

4. A complex according to any one of claims 1, 2 or 3, comprising a functional double stranded T3, T7 or SP6 RNA polymerase promoter.

5. A complex according to any one of the preceding claims, comprising single or double stranded sequence adjacent to the promoter which increases the activity of the promoter.

6. A complex according to claim 5, wherein one of said probes comprises a +12 sequence.

7. A complex according to claim 5, wherein the first probe comprises a +12 sequence.

8. A complex according to any one of the preceding claims, comprising a sequence which, when transcribed into RNA, facilitates isolation, identification, detection, quantification or amplification of the transcript.

9. A complex according to any one of the preceding claims, wherein one of said probes comprises a destabilizing moiety.

10. A complex according to any one of the preceding claims, wherein the second and third probes form a discontinuous sequence of an RNA polymerase promoter template strand.

11. A complex according to any one of claims 1-9, wherein the second and third probes form a discontinuous sequence of an RNA polymerase promoter non-template strand.

12. A method of detecting the presence of a target nucleic acid molecule in a sample, the method comprising the steps of: contacting the sample comprising the target with first and second nucleic acid probes, each probe comprising a foot region complementary to respective first and second portions of the target, which portions are adjacent or substantially so; wherein the first and second probes each further comprise an arm region substantially non-complementary to the target, at least part of the arm region of

the first probe being complementary to at least part of the arm region of the second probe, such that respective foot regions of the first and second probes hybridise to the target, allowing hybridisation of the complementary parts of the arm regions of the first and second probes; and causing to be present a third nucleic acid probe molecule which is complementary to a portion of the arm region of the first probe, such that the third probe hybridises to the first probe adjacent or substantially adjacent to the arm region of the second probe, thereby creating a functional double-stranded RNA polymerase promoter, one strand of the promoter being provided by the first probe, the other strand being provided jointly by the second and third probes; causing RNA synthesis from the RNA promoter; and detecting, directly or indirectly, the RNA so synthesised.

13. A method according to claim 12, performance of which results in the formation of a complex in accordance with any one of claims 1-11.

14. A method according to claim 12 or 13, wherein RNA produced from the functional RNA promoter is amplified prior to detection.

15. A method according to any one of claims 12, 13 or 14, wherein RNA produced from the functional RNA promoter is detected directly or indirectly via a method which involves use of a molecular beacon or fluorophore.

16. A kit of use in performing the method of claim 12, comprising a first probe and a third probe molecule as defined in claim 1.

17. A kit of use in performing the method of claim 12, comprising a second probe and a third probe molecule as defined in claim 1.

18. A kit of use in performing the method of claim12, comprising a first, second and third probe molecule as defined in claim 1.

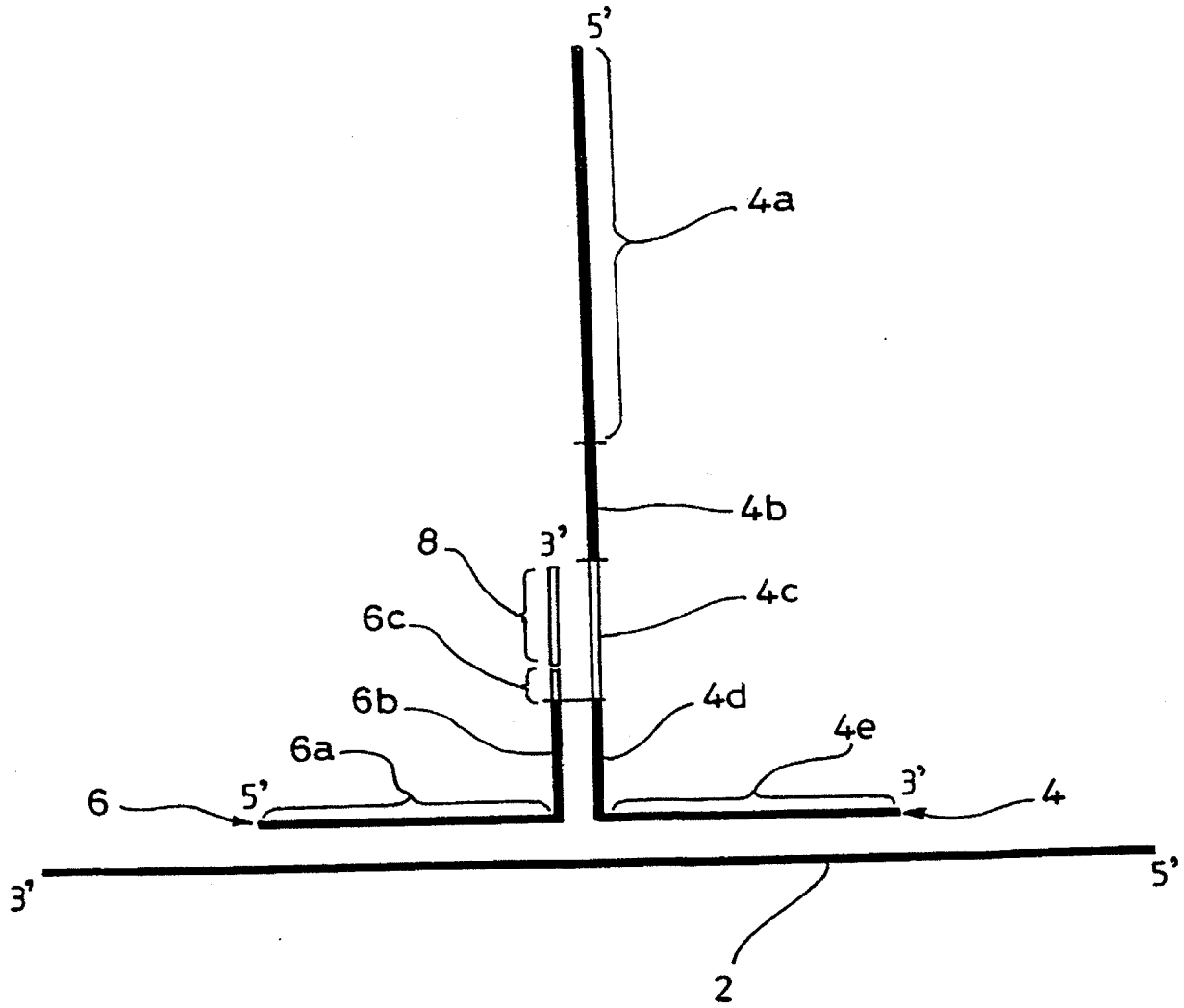


Fig. 1

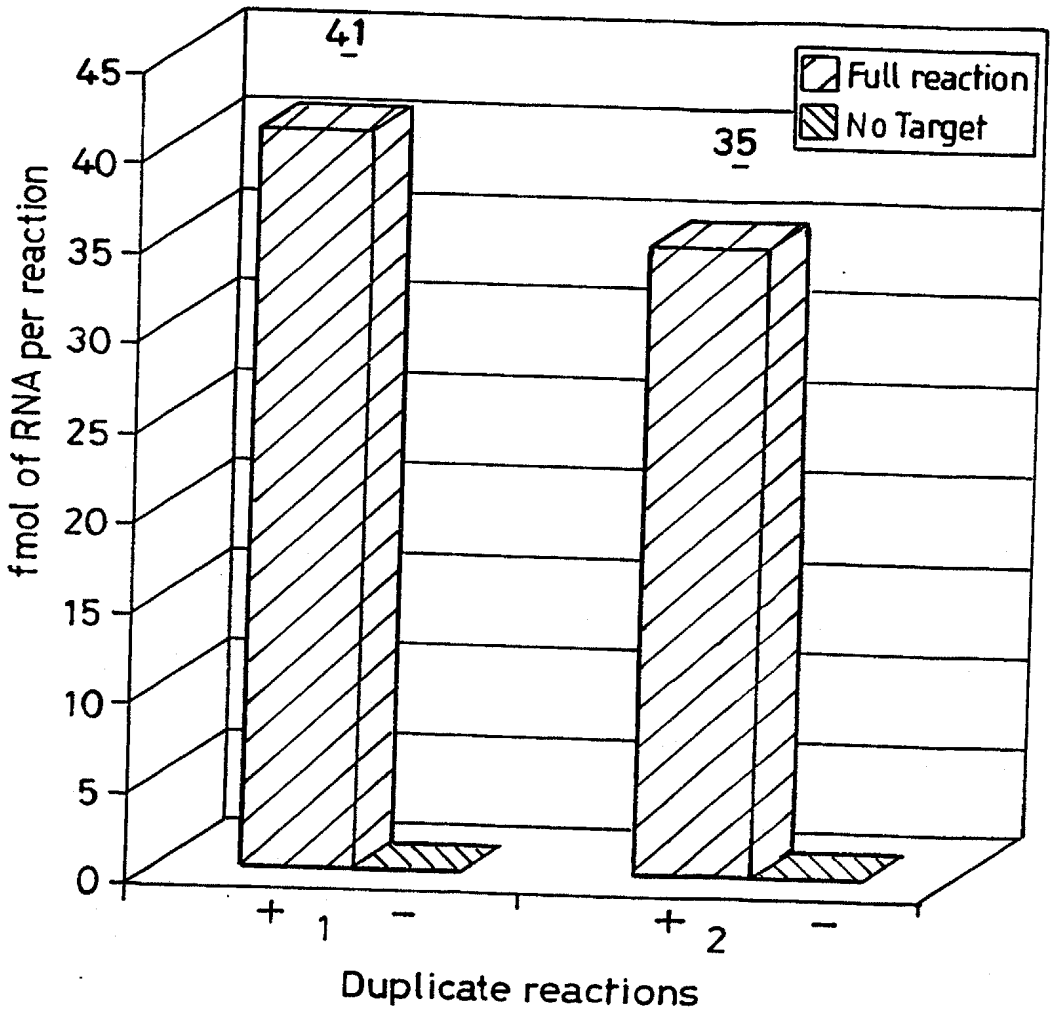

41
35
Full reaction
No Target
45
40
35
30
25
20
15
10
5
0
fmol of RNA per reaction
+ 1 −
+ 2 −
Duplicate reactions

Fig. 2

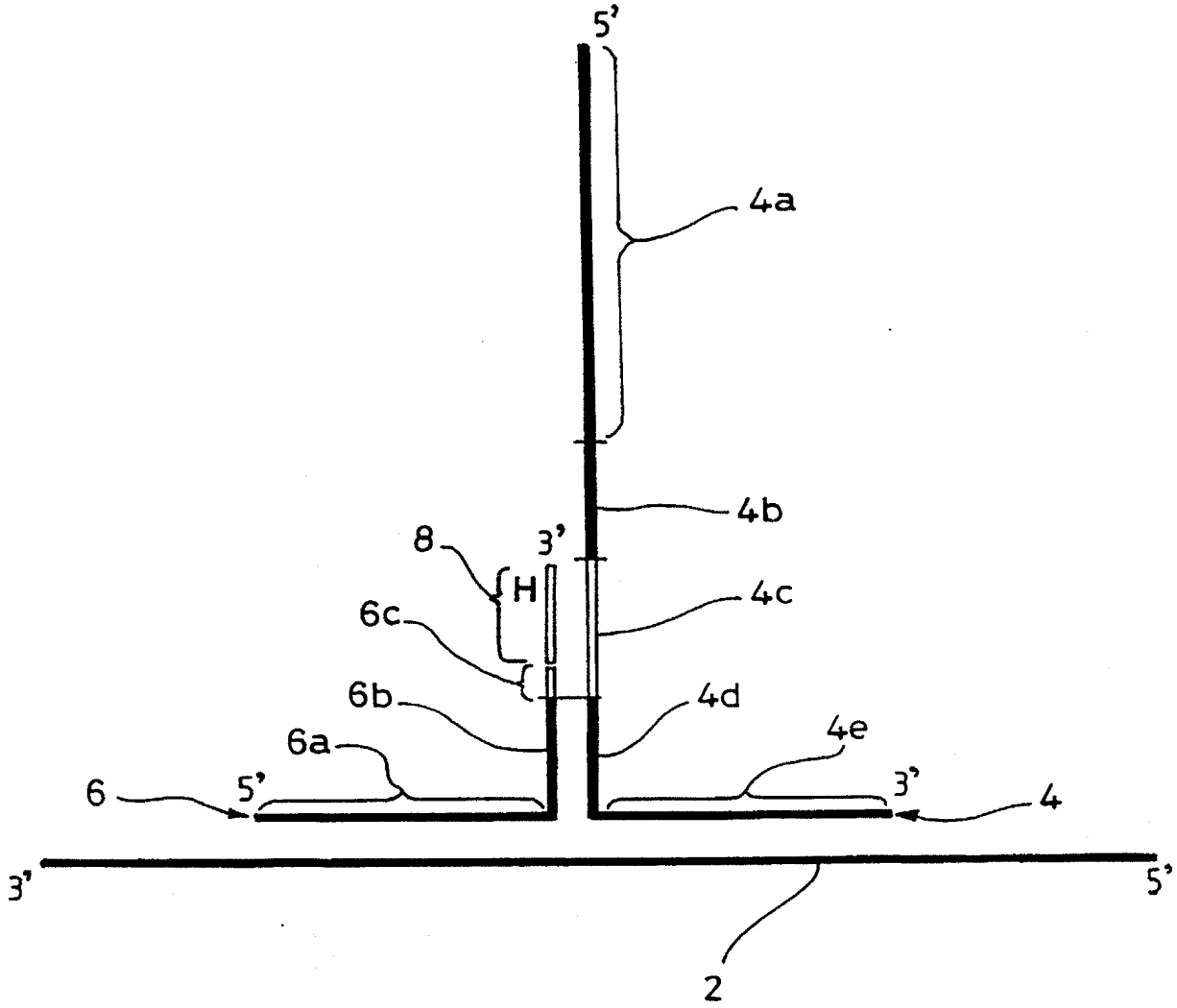


Fig. 3

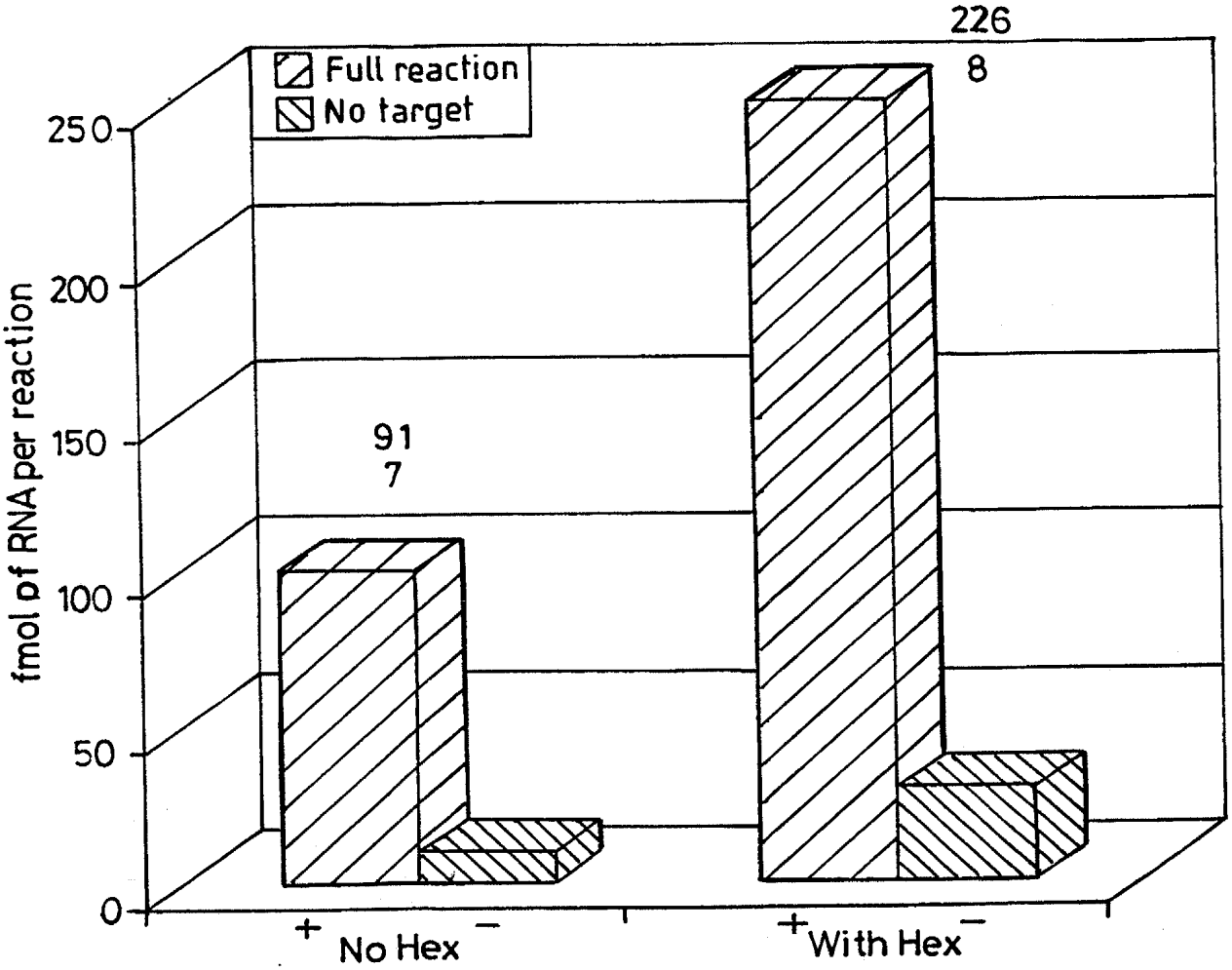

Full reaction
No target
fmol of RNA per reaction
250
200
150
100
50
0
91
7
226
8
+ No Hex −
+ With Hex −

Fig. 4

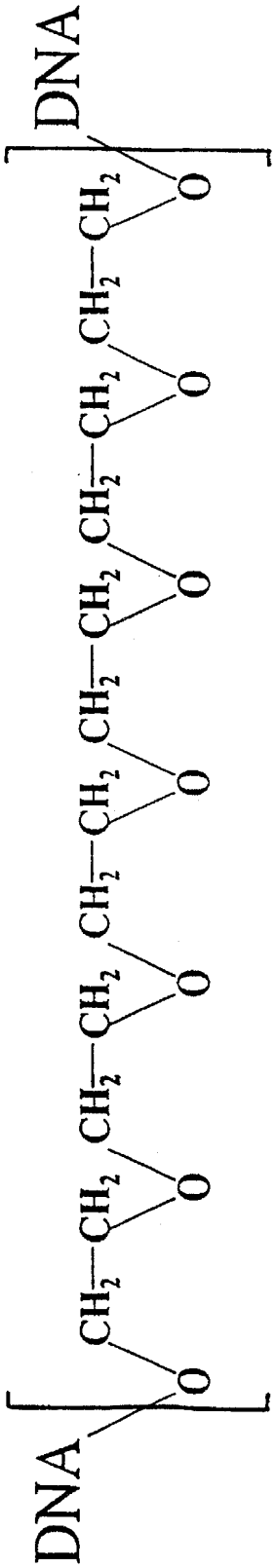


Fig. 5

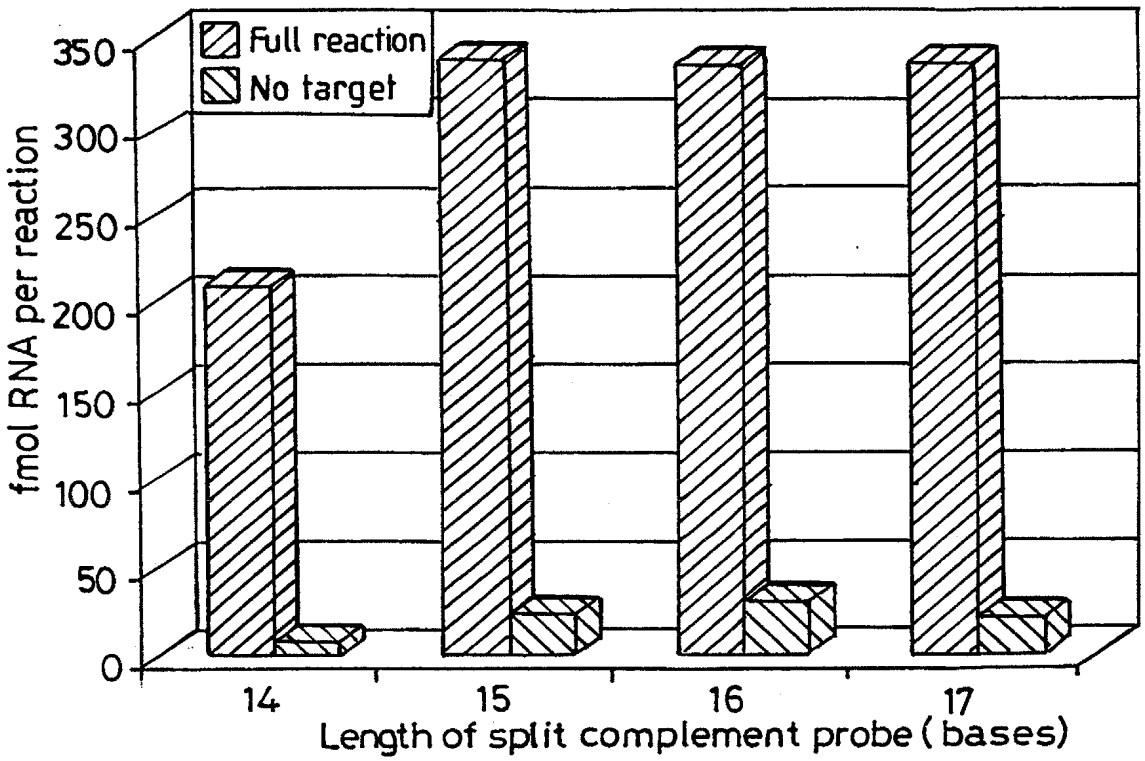

Full reaction
No target
350
300
250
200
150
100
50
0
fmol RNA per reaction
14
15
16
17
Length of split complement probe (bases)

Fig. 6

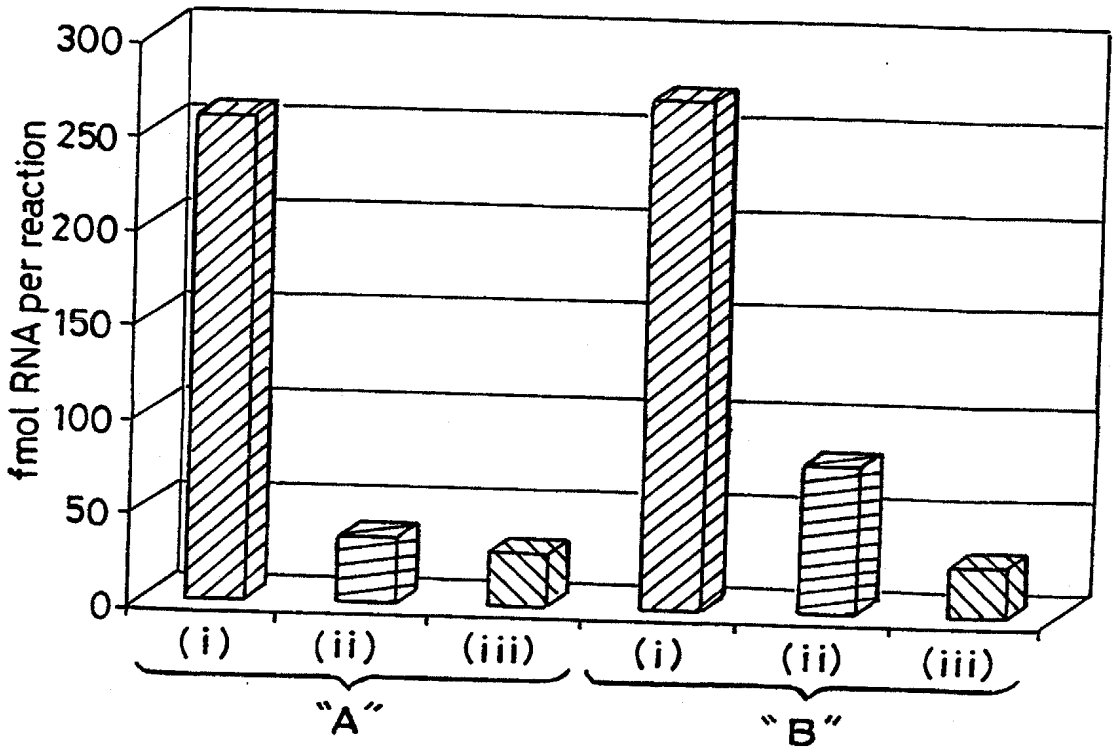

300
250
200
150
100
50
0
fmol RNA per reaction
(i)
(ii)
(iii)
(i)
(ii)
(iii)
"A"
"B"

Fig. 7

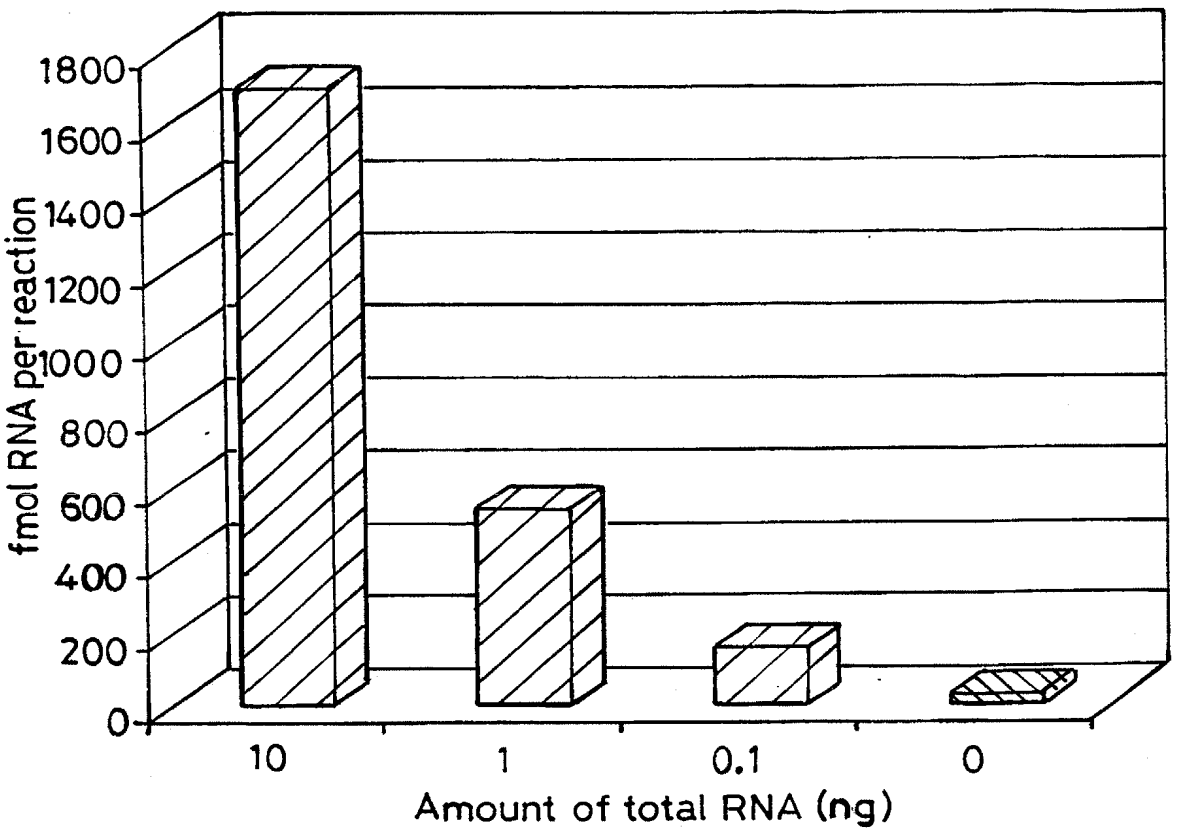

1800
1600
1400
1200
1000
800
600
400
200
0
fmol RNA per reaction
10
1
0.1
0
Amount of total RNA (ng)

Fig. 8

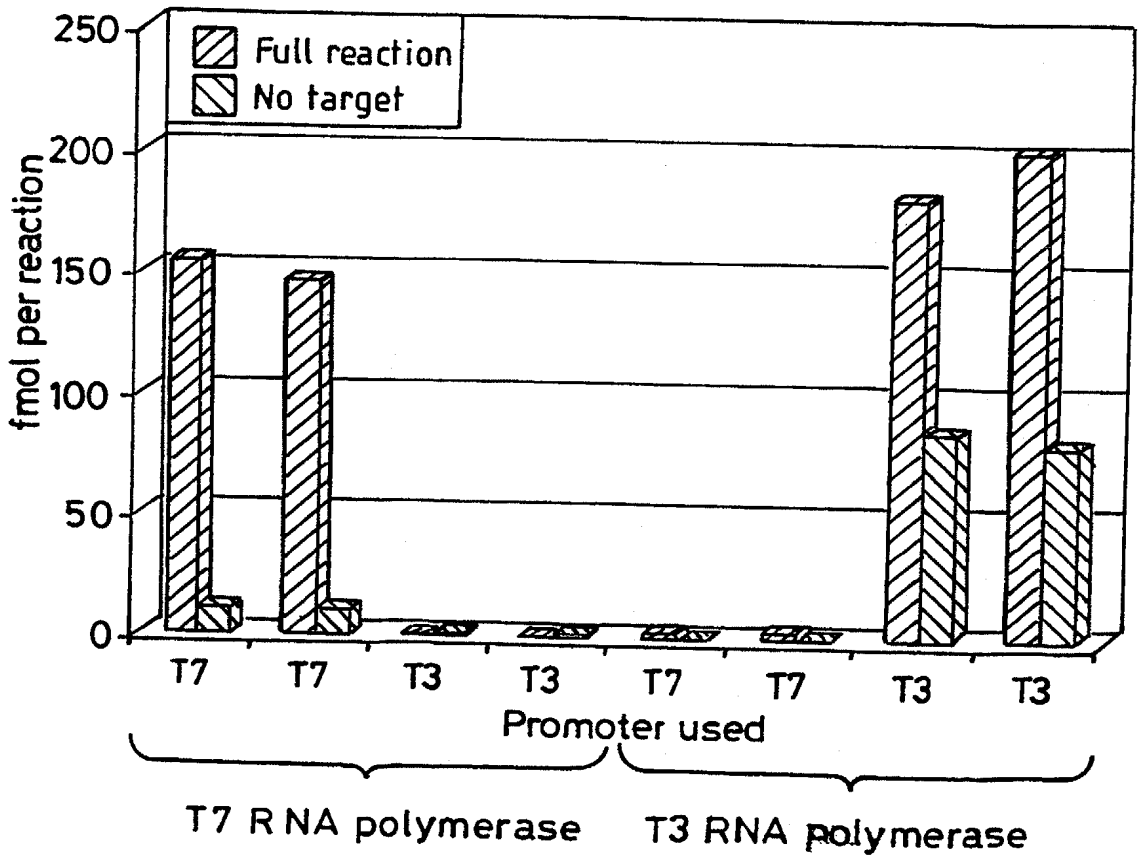

250
200
150
100
50
0
fmol per reaction
Full reaction
No target
T7
T7
T3
T3
T7
T7
T3
T3
Promoter used
T7 RNA polymerase
T3 RNA polymerase

Fig. 9

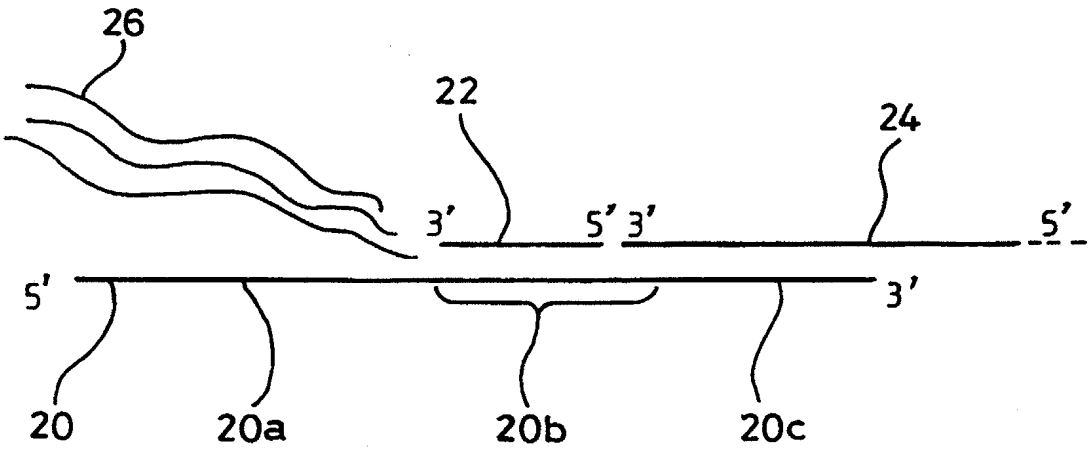


Fig. 10

SEQUENCE LISTING

<110> Cytocell Limited

<120> Improvements in or Relating to Nucleic Acid Assays

<130> MJL/C1092'1/C

<140>
<141>

<160> 39

<170> PatentIn Ver. 2.1

<210> 1
<211> 18
<212> DNA
<213> Bacteriophage T3

<400> 1
aaattaaccc tcactaaa 18

<210> 2
<211> 18
<212> DNA
<213> Bacteriophage T3

<400> 2
ttattaaccc tcactaaa 18

<210> 3
<211> 17
<212> DNA
<213> Bacteriophage T7

<400> 3
taatacgact cactata 17

<210> 4
<211> 17
<212> DNA
<213> Bacteriophage SP6

<400> 4

INTERNATIONAL SEARCH REPORT

Interr. Application No
PCT/GB 00/02962

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	WO 99 37805 A (CARDY DONALD LEONARD NICHOLAS ;RAY TREVOR DUNCAN (GB); MARSH PETER) 29 July 1999 (1999-07-29) page 6, paragraph 6; figures 1,2 page 9, paragraph 2 -page 10, paragraph 3 page 7, paragraph 2 -page 8, paragraph 2 ---	1-17
P,Y	WO 99 37806 A (CARDY DONALD LEONARD NICHOLAS ;RAY TREVOR DUNCAN (GB); MARSH PETER) 29 July 1999 (1999-07-29) page 4, paragraph 2; figures 1,9,11A,11B,13A,14A; examples 6,9 ---	1-17
A	EP 0 851 033 A (GEN PROBE INC) 1 July 1998 (1998-07-01) page 10, line 31 -page 13, line 3 ---	1
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 November 2000	28/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern[illegible]pplication No
PCT/GB 00/02962

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93 06240 A (CYTOCELL LTD) 1 April 1993 (1993-04-01) figure 2	1
A	EP 0 552 931 A (GEN PROBE INC) 28 July 1993 (1993-07-28) figure 15C	1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr. Application No
PCT/GB 00/02962

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9937805 A	29-07-1999	AU 2431799 A	09-08-1999
		EP 1049806 A	08-11-2000
WO 9937806 A	29-07-1999	AU 2289299 A	09-08-1999
		EP 1051515 A	15-11-2000
EP 0851033 A	01-07-1998	US 6025133 A	15-02-2000
		AU 5525998 A	31-07-1998
		WO 9829568 A	09-07-1998
WO 9306240 A	01-04-1993	AT 152778 T	15-05-1997
		AU 672367 B	03-10-1996
		AU 2558692 A	27-04-1993
		CA 2118913 A	01-04-1993
		DE 69219627 D	12-06-1997
		DE 69219627 T	04-09-1997
		DK 666927 T	15-09-1997
		EP 0666927 A	16-08-1995
		ES 2101116 T	01-07-1997
		JP 6510669 T	01-12-1994
EP 0552931 A	28-07-1993	AU 665062 B	14-12-1995
		AU 3586693 A	01-09-1993
		CA 2128530 A	05-08-1993
		DE 69328699 D	29-06-2000
		DE 69328699 T	07-09-2000
		JP 7503139 T	06-04-1995
		WO 9315102 A	05-08-1993
		US 5451503 A	19-09-1995
		US 5424413 A	13-06-1995

atttaggtga cactata 17

<210> 5
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:synthetic oligonucleotide

<400> 5
atcgtcagtc cc 12

<210> 6
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 6
gctctctctc cc 12

<210> 7
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 7
atcctctctc cc 12

<210> 8
<211> 12
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 8
gttctctctc cc 12

<210> 9
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 9
gatgtgtctc cc 12

<210> 10
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 10
gttgtgtctc cc 12

<210> 11
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 11
atcctcgtgc cc 12

<210> 12
<211> 12

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 12
gctctcgtgc cc 12

<210> 13
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 13
gttctcgtgc cc 12

<210> 14
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 14
gttgtggtgc cc 12

<210> 15
<211> 108
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 15
tgcctccttg tctccgttct ggatatcacc cgatgtggct ctctctccct atagtgagtc 60

gtattaattt cgaaggtgtt tcctatgatg aatatagata cagaagcg 108

<210> 16
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 16
gcctggcacc attaaagaaa atatcatctt tttcgaaatt aa 42

<210> 17
<211> 14
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 17
tacgactcac tata 14

<210> 18
<211> 120
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 18
gttggcatgc tttgatgacg cttctgtatc tatattcatc ataggaaaca ccaaagatga 60
tattttcttt aatggtgcca ggcataatcc aggaaaactg agaacagaat gaaattcttc 120

<210> 19
<211> 49
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: RNA transcript
of synthetic oligonucleotide

<400> 19
gggagagaga gccacaucgg gugauaucca gaacggagac aaggaggca 49

<210> 20
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 20
tctgctgcct gcttgtctgc gttct 25

<210> 21
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 21
ggatatcacc cg 12

<210> 22
<211> 103
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 22
tcgtcttccg gtctctcctc tcaagcctca gcgctctctc tccctatagt gagtcgtatt 60
aatttcgaag gtgtttccta tgatgaatat agatacagaa gcg 103

<210> 23
<211> 44
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: RNA transcrip
of synthetic oligonucleotide

<400> 23
gggagagaga gcgcugaggc uugagaggag agaccggaag acga

<210> 24
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 24
tctgctcgtc ttccggtctc tcctc

<210> 25
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 25
tcaagcctca gc

<210> 26
<211> 83
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 26
tcgtcttccg gtctctcctc tcaagcctca gcgctctctc tccctatagt gagtcgtatt 60
aatttcgaaa tatcatcttt ggt 83

<210> 27
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 27
tcgtcttccg gtctctcctc tcaagcctca gcgctctctc tccctatagt gagtcgtatt 60
aatttcgaaa tatcatcttt ggtgtttcct atgatgaat 99

<210> 28
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 28
gcctggcacc attaaagaaa ttcgaaatta a 31

<210> 29
<211> 77
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 29
gatgacgctt ctgtatctat attcatcata ggaaacacca atgatatttt ctttaatggt 60
gccaggcata atccagg 77

<210> 30
<211> 95

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 30
tcgtcttccg gtctctcctc tcaagcctca gcgctctctc tccctatagt gagtcgtatt 60
aatttcgaag gcatgacaac ccgaacacca gtgat 95

<210> 31
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 31
gcatttagct accgggcagt gccattttcg aaattaa 37

<210> 32
<211> 111
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 32
tgcctgcttg tctgcgttct ggatatcacc cgagttctcg cttcctatag tgagtcgtat 60
taatttctcg tcttccggtc tctcctctca agcctcagcg ctctctctcc c 111

<210> 33
<211> 45
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: RNA transcript
of synthetic oligonucleotide

<400> 33
ggaagcgaga acucggguga uauccagaac gcagacaagc aggca 45

<210> 34
<211> 103
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 34
tcgtcttccg gtctctcctc tcaagcctca gccttctctc ttcctatagt gagtcgtatt 60
aatttcgaag gtgtttccta tgatgaatat agatacagaa gcg 103

<210> 35
<211> 121
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 35
cctcctctag ttggcatgct ttgatgacgc ttctgtatct atattcatca taggaaacac 60
caaagatgat attttcttta atggtgccag gcataatcca ggaaaactga gaacagaatg 120
a 121

<210> 36
<211> 103
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 36
tcgtcttccg gtctctcctc tcaagcctca gccttctctc ttcctttagt gagggttaat 60
tatttcgaag gtgtttccta tgatgaatat agatacagaa gcg 103

<210> 37

<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 37
gcctggcacc attaaagaaa atatcatctt tttcgaaata at 42

<210> 38
<211> 14
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 38
taaccctcac taaa 14

<210> 39
<211> 44
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: RNA transcript of synthetic oligonucleotide

<400> 39
ggaagagaga aggcugaggc uugagaggag agaccggaag acga 44

SEQUENCE LISTING

<110> Cytocell Limited

<120> Improvements in or Relating to Nucleic Acid Assays

<130> 056222-5009-US

<140>
<141>

<150> PCT/GB00/02962
<151> 2000-07-31

<160> 39

<170> PatentIn Ver. 2.1

<210> 1
<211> 18
<212> DNA
<213> Bacteriophage T3

<400> 1
aaattaaccc tcactaaa 18

<210> 2
<211> 18
<212> DNA
<213> Bacteriophage T3

<400> 2
ttattaaccc tcactaaa 18

<210> 3
<211> 17
<212> DNA
<213> Bacteriophage T7

<400> 3
taatacgact cactata 17

<210> 4
<211> 17
<212> DNA
<213> Bacteriophage SP6

<400> 4
atttaggtga cactata 17

<210> 5
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:synthetic

oligonucleotide

<400> 5
atcgtcagtc cc 12

<210> 6
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 6
gctctctctc cc 12

<210> 7
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 7
atcctctctc cc 12

<210> 8
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 8
gttctctctc cc 12

<210> 9
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 9
gatgtgtctc cc 12

<210> 10
<211> 12
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 10
gttgtgtctc cc 12

<210> 11
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 11
atcctcgtgc cc 12

<210> 12
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 12
gctctcgtgc cc 12

<210> 13
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 13
gttctcgtgc cc 12

<210> 14
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 14
gttgtggtgc cc 12

<210> 15
<211> 108
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 15
tgcctccttg tctccgttct ggatatcacc cgatgtggct ctctctccct atagtgagtc 60
gtattaattt cgaaggtgtt tcctatgatg aatatagata cagaagcg 108

<210> 16
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 16
gcctggcacc attaaagaaa atatcatctt tttcgaaatt aa 42

<210> 17
<211> 14
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 17
tacgactcac tata 14

<210> 18
<211> 120
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 18
gttggcatgc tttgatgacg cttctgtatc tatattcatc ataggaaaca ccaaagatga 60
tattttcttt aatggtgcca ggcataatcc aggaaaactg agaacagaat gaaattcttc 120

<210> 19
<211> 49
<212> RNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: RNA transcript
of synthetic oligonucleotide

<400> 19
gggagagaga gccacaucgg gugauaucca gaacggagac aaggaggca 49

<210> 20
<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 20
tctgctgcct gcttgtctgc gttct 25

<210> 21
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 21
ggatatcacc cg 12

<210> 22
<211> 103
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 22
tcgtcttccg gtctctcctc tcaagcctca gcgctctctc tccctatagt gagtcgtatt 60
aatttcgaag gtgtttccta tgatgaatat agatacagaa gcg 103

<210> 23
<211> 44
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: RNA transcript
of synthetic oligonucleotide

<400> 23
gggagagaga gcgcugaggc uugagaggag agaccggaag acga 44

<210> 24

<211> 25
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 24
tctgctcgtc ttccggtctc tcctc 25

<210> 25
<211> 12
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 25
tcaagcctca gc 12

<210> 26
<211> 83
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 26
tcgtcttccg gtctctcctc tcaagcctca gcgctctctc tccctatagt gagtcgtatt 60
aatttcgaaa tatcatcttt ggt 83

<210> 27
<211> 99
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 27
tcgtcttccg gtctctcctc tcaagcctca gcgctctctc tccctatagt gagtcgtatt 60
aatttcgaaa tatcatcttt ggtgtttcct atgatgaat 99

<210> 28
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 28
gcctggcacc attaaagaaa ttcgaaatta a 31

<210> 29
<211> 77
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 29
gatgacgctt ctgtatctat attcatcata ggaaacacca atgatatttt ctttaatggt 60
gccaggcata atccagg 77

<210> 30
<211> 95
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 30
tcgtcttccg gtctctcctc tcaagcctca gcgctctctc tccctatagt gagtcgtatt 60
aatttcgaag gcatgacaac ccgaacacca gtgat 95

<210> 31
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 31
gcatttagct accgggcagt gccattttcg aaattaa 37

<210> 32
<211> 111
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 32
tgcctgcttg tctgcgttct ggatatcacc cgagttctcg cttcctatag tgagtcgtat 60
taatttctcg tcttccggtc tctcctctca agcctcagcg ctctctctcc c 111

<210> 33

<211> 45
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: RNA transcript
of synthetic oligonucleotide

<400> 33
ggaagcgaga acucggguga uauccagaac gcagacaagc aggca 45

<210> 34
<211> 103
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 34
tcgtcttccg gtctctcctc tcaagcctca gccttctctc ttcctatagt gagtcgtatt 60
aatttcgaag gtgtttccta tgatgaatat agatacagaa gcg 103

<210> 35
<211> 121
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 35
cctcctctag ttggcatgct ttgatgacgc ttctgtatct atattcatca taggaaacac 60
caaagatgat attttcttta atggtgccag gcataatcca ggaaaactga gaacagaatg 120
a 121

<210> 36
<211> 103
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic
oligonucleotide

<400> 36
tcgtcttccg gtctctcctc tcaagcctca gccttctctc ttcctttagt gagggttaat 60
tatttcgaag gtgtttccta tgatgaatat agatacagaa gcg 103

<210> 37
<211> 42
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 37
gcctggcacc attaaagaaa atatcatctt tttcgaaata at 42

<210> 38
<211> 14
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: synthetic oligonucleotide

<400> 38
taaccctcac taaa 14

<210> 39
<211> 44
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: RNA transcript of synthetic oligonucleotide

<400> 39
ggaagagaga aggcugaggc uugagaggag agaccggaag acga 44

INTERNATIONAL SEARCH REPORT

Intern. Application No
PCT/GB 00/02962

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	WO 99 37805 A (CARDY DONALD LEONARD NICHOLAS ;RAY TREVOR DUNCAN (GB); MARSH PETER) 29 July 1999 (1999-07-29) page 6, paragraph 6; figures 1,2 page 9, paragraph 2 -page 10, paragraph 3 page 7, paragraph 2 -page 8, paragraph 2	1-17
P,Y	WO 99 37806 A (CARDY DONALD LEONARD NICHOLAS ;RAY TREVOR DUNCAN (GB); MARSH PETER) 29 July 1999 (1999-07-29) page 4, paragraph 2; figures 1,9,11A,11B,13A,14A; examples 6,9	1-17
A	EP 0 851 033 A (GEN PROBE INC) 1 July 1998 (1998-07-01) page 10, line 31 -page 13, line 3	1

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

°A° document defining the general state of the art which is not considered to be of particular relevance

°E° earlier document but published on or after the international filing date

°L° document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

°O° document referring to an oral disclosure, use, exhibition or other means

°P° document published prior to the international filing date but later than the priority date claimed

°T° later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

°X° document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

°Y° document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

°&° document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 November 2000	28/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Osborne, H

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. Application No
PCT/GB 00/02962

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9937805 A	29-07-1999	AU 2431799 A	09-08-1999
		EP 1049806 A	08-11-2000
WO 9937806 A	29-07-1999	AU 2289299 A	09-08-1999
		EP 1051515 A	15-11-2000
EP 0851033 A	01-07-1998	US 6025133 A	15-02-2000
		AU 5525998 A	31-07-1998
		WO 9829568 A	09-07-1998
WO 9306240 A	01-04-1993	AT 152778 T	15-05-1997
		AU 672367 B	03-10-1996
		AU 2558692 A	27-04-1993
		CA 2118913 A	01-04-1993
		DE 69219627 D	12-06-1997
		DE 69219627 T	04-09-1997
		DK 666927 T	15-09-1997
		EP 0666927 A	16-08-1995
		ES 2101116 T	01-07-1997
		JP 6510669 T	01-12-1994
EP 0552931 A	28-07-1993	AU 665062 B	14-12-1995
		AU 3586693 A	01-09-1993
		CA 2128530 A	05-08-1993
		DE 69328699 D	29-06-2000
		DE 69328699 T	07-09-2000
		JP 7503139 T	06-04-1995
		WO 9315102 A	05-08-1993
		US 5451503 A	19-09-1995
		US 5424413 A	13-06-1995

PATENT COOPERATION TREAT[redacted]

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION CONCERNING SUBMISSION OR TRANSMITTAL OF PRIORITY DOCUMENT

(PCT Administrative Instructions, Section 411)

To:

KEITH W NASH & CO
90-92 Regent Street
Cambridge CB2 1DP
ROYAUME-UNI

Date of mailing (day/month/year) 12 September 2000 (12.09.00)	
Applicant's or agent's file reference MJL/1092.2/C	**IMPORTANT NOTIFICATION**
International application No. PCT/GB00/02962	International filing date (day/month/year) 31 July 2000 (31.07.00)
International publication date (day/month/year) Not yet published	Priority date (day/month/year) 29 July 1999 (29.07.99)
Applicant CYTOCELL LIMITED et al	

1. The applicant is hereby notified of the date of receipt (except where the letters "NR" appear in the right-hand column) by the International Bureau of the priority document(s) relating to the earlier application(s) indicated below. Unless otherwise indicated by an asterisk appearing next to a date of receipt, or by the letters "NR", in the right-hand column, the priority document concerned was submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b).

2. This updates and replaces any previously issued notification concerning submission or transmittal of priority documents.

3. An asterisk(*) appearing next to a date of receipt, in the right-hand column, denotes a priority document submitted or transmitted to the International Bureau but not in compliance with Rule 17.1(a) or (b). In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

4. The letters **"NR"** appearing in the right-hand column denote a priority document which was not received by the International Bureau or which the applicant did not request the receiving Office to prepare and transmit to the International Bureau, as provided by Rule 17.1(a) or (b), respectively. In such a case, **the attention of the applicant is directed** to Rule 17.1(c) which provides that no designated Office may disregard the priority claim concerned before giving the applicant an opportunity, upon entry into the national phase, to furnish the priority document within a time limit which is reasonable under the circumstances.

Priority date	Priority application No.	Country or regional Office or PCT receiving Office	Date of receipt of priority document
29 July 1999 (29.07.99)	9917813.9	GB	23 Augu 2000 (23.08.00)
17 Augu 1999 (17.08.99)	60/149,176	US	23 Augu 2000 (23.08.00)

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No. (41-22) 740.14.35

Authorized officer

R. Raissi

Telephone No. (41-22) 338.83.38

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
KEITH W NASH & CO
90-92 Regent Street
Cambridge CB2 1DP
ROYAUME-UNI

Date of mailing (day/month/year) 08 February 2001 (08.02.01)	
Applicant's or agent's file reference MJL/1092.2/C	**IMPORTANT NOTICE**
International application No. PCT/GB00/02962	**International filing date** (day/month/year) 31 July 2000 (31.07.00)
Priority date (day/month/year) 29 July 1999 (29.07.99)	
Applicant CYTOCELL LIMITED et al	

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   CA,EP,JP

   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   08 February 2001 (08.02.01) under No. WO 01/09377

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a **demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer J. Zahra
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

Form PCT/IB/308 (July 1996) 3810100

(12) INTERNATIONAL APP[illegible]TION PUBLISHED UNDER THE PATENT [illegible]PERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
8 February 2001 (08.02.2001)

PCT

(10) International Publication Number
WO 01/09377 A1

(51) International Patent Classification[7]: C12Q 1/68

(21) International Application Number: PCT/GB00/02962

(22) International Filing Date: 31 July 2000 (31.07.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9917813.9 29 July 1999 (29.07.1999) GB
60/149,176 17 August 1999 (17.08.1999) US

(71) Applicant *(for all designated States except US)*: **CYTOCELL LIMITED** [GB/GB]; Unit 6, Somerville Court, Trinity Way, Adderbury, Banbury, Oxfordshire OX17 3SN (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **LLOYD, John, Scott** [GB/GB]; 14 Windsor Close, King Sutton, Oxfordshire OX17 3QT (GB). **WESTON, Anthony** [GB/GB]; 8 New Court, Dorchester Close, Northolt, Middlesex UB5 4PF (GB). **CARDY, Donald, Leonard, Nicholas** [GB/GB]; Trinlan, Blacksmith's Lane, Aston-le-Walls, Northamptonshire NN11 6UN (GB).

(74) Agent: **KEITH W NASH & CO**; 90-92 Regent Street, Cambridge CB2 1DP (GB).

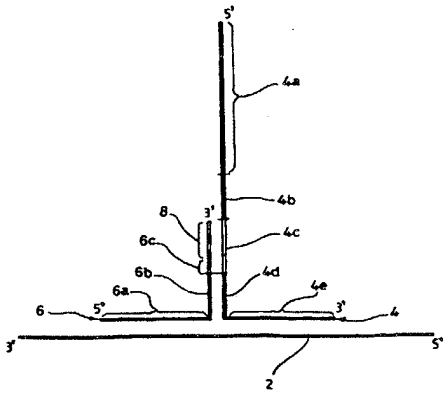
(81) Designated States *(national)*: AU, CA, JP, US.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD FOR DETECTING NUCLEIC ACID TARGET SEQUENCES INVOLVING IN VITRO TRANSCRIPTION FROM AN RNA POLYMERASE PROMOTER



(57) Abstract: Disclosed is a complex formed by a hybridisation reaction comprising four nucleic acid molecules; the complex comprising a target nucleic acid molecule and first, second and third nucleic acid probe molecules; wherein the first probe comprises a foot region which is complementary to a first portion of the target and is hybridised thereto, and an arm region which is substantially non-complementary to the target; the second probe comprises a foot region which is complementary to a second portion of the target, such that the foot region of the second probe is hybridised to the target adjacent or substantially adjacent to the foot region of the first probe, the second probe also comprising an arm region which is substantially non-complementary to the target but which is complementary and hybridised to the arm region of the first probe; the third probe being complementary, at least in part, to a portion of the arm region of the first probe, such that third probe is hybridised to the arm region of the first probe adjacent or substantially adjacent to the second probe; and wherein formation of the complex creates a functional double-stranded RNA polymerase promoter, one strand of the promoter being provided by the first probe, and the other strand being provided jointly by the second probe and by the third probe; and a method of detecting a target nucleic acid sequence of interest which method involves the formation of the complex.

WO 01/09377 A1

10/prts

METHOD FOR DETECTING NUCLEIC ACID TARGET SEQUENCES INVOLVING IN VITRO TRANSCRIPTION FROM AN RNA POLYMERASE PROMOTER

Field of the Invention

This invention relates to nucleic acid complexes comprising a functional RNA polymerase promoter, and to a method of detecting a target nucleic acid sequence of interest.

Background of the Invention

RNA polymerases are enzyme molecules well-known to those skilled in the fields of molecular biology and molecular diagnostic kits. RNA polymerases synthesise RNA molecules from a DNA template strand.

Much research has been carried out on RNA polymerases, especially bacteriophage RNA polymerases.

Specifically, the RNA polymerase from the bacteriophage T7 has been shown to be very selective for specific promoters that are rarely encountered in DNA unrelated to T7 DNA (Chamberlin *et al*, 1970 Nature **228**, 227; Dunn & Studier 1983 J. Mol. Biol. **166**, 477). T7 RNA polymerase is able to make complete transcripts of almost any DNA that is placed under control of a T7 promoter. T7 RNA polymerase is a highly active enzyme that transcribes about five times faster than does *Escherichia coli* RNA polymerase (Studier *et al*, 1990 Methods Enzymol. **185**, 60). The synthesis of small RNAs using T7 RNA polymerase has been described whereby sequences around the RNA polymerase promoter sequence are shown to be important in the reproducible improvement of yield of RNA produced (Milligan & Uhlenbeck, 1989 Methods Enzymol. **180**, 51 and Milligan *et al*, 1987 Nucl. Acids Res. **15**, 8783-8798). Other RNA polymerases that have similar properties to T7 include those from bacteriophage T3 and SP6, the genes for which have all been cloned and the corresponding enzymes are commercially available. The optimum promoter sequences for T7, T3 and SP6 polymerases are known, and are essentially 17 nucleotides long.

A number of methods have been disclosed, which utilise RNA polymerases to synthesise RNA directly or indirectly as the result of the presence of a particular nucleic acid sequence of interest ("target"). The presence of RNA (detected directly or indirectly) thus serves to signal the presence of the sequence of interest and can be used as the basis of assay methods and/or diagnostic methods or kits. Examples include the disclosures of WO 93/06240, WO 94/29481, EP 0851033, and EP 0552931.

In particular WO 93/06240 discloses the use of two probes, which hybridise together only in the presence of a target nucleic acid sequence of interest, such that hybridisation of the probes to each other is indicative of the presence of the sequence of interest.

All publications mentioned in this specification are incorporated herein by reference.

Summary of the Invention

In a first aspect the invention provides a complex formed by a hybridisation reaction comprising four nucleic acid molecules; the complex comprising a target nucleic acid molecule and first, second and third nucleic acid probe molecules; wherein the first probe comprises a foot region which is complementary to a first portion of the target and is hybridised thereto, and an arm region which is substantially non-complementary to the target; the second probe comprises a foot region which is complementary to a second portion of the target, such that the foot region of the second probe is hybridised to the target adjacent or substantially adjacent to the foot region of the first probe, the second probe also comprising an arm region which is substantially non-complementary to the target but which is complementary and hybridised to the arm region of the first probe; the third probe being complementary, at least in part, to a portion of the arm region of the first probe, such that the third probe is hybridised to the arm region of the first probe adjacent or substantially adjacent to the second probe; and wherein formation of the complex creates a functional double-stranded RNA polymerase promoter, one strand of the promoter being provided by the first probe, and the other strand being provided jointly by the second probe and by the third probe.

Preferably the sequence of the probes and the hybridisation reaction conditions are selected such that the first and second probes cannot become hybridised in the absence of the target, such that formation of the RNA polymerase promoter occurs in a target-dependent manner.

In a second aspect, the invention provides a method of detecting the presence of a target nucleic acid molecule in a sample, the method comprising the steps of contacting the sample comprising the target with first and second nucleic acid probes, each probe comprising a foot region complementary to respective first and second portions of the target, which portions are adjacent or substantially so; wherein the first and second probes each further comprise an arm region substantially non-complementary to the target, at least part of the arm region of the first probe being complementary to at least part of the arm region of the second probe, such that respective foot regions of the first and second probes hybridise to the target, allowing hybridisation of the complementary parts of the arm regions of the first and second probes; and causing to be present a third nucleic acid probe molecule which is complementary to a portion of the arm region of the first probe, such that the third probe hybridises to the first probe adjacent or substantially adjacent to the arm region of the second probe, thereby creating a functional double-stranded RNA polymerase promoter, one strand of the promoter being provided by the first probe, the other strand being provided jointly by the second and third probes; causing RNA synthesis from the RNA promoter; and detecting, directly or indirectly, the RNA so synthesised.

The method of the second aspect of the invention thus results in formation of the complex of the first aspect.

It is an essential feature of the invention that the first and second probes, when hybridised to the target sequence, are adjacent or substantially adjacent to each other. Use of the term "adjacent" is herein intended to mean that there are no nucleotides of the target sequence left without base-pairing between those portions of the target sequence which are base-paired to the complementary sequence of the probes. This proximity between the probes

enables the complementary arm portions of the probes to anneal. As will readily be apparent to those skilled in the art, by designing the probes so as to allow for annealing to each other at greater separations from the target sequence, gaps may be introduced between the loci in the target nucleotide sequence to which the probes hybridise. In this situation the probes are said to be "substantially adjacent", because there may be some nucleotides of the target sequence left without base-pairing between those portions of the target sequence which are base-paired to the probes. Clearly, the number of intervening un-paired nucleotides of the target sequence can vary according to the design of the probes. Thus whilst it is preferred that the first and second probes hybridise so as to be adjacent, the probes may be separated by up to 5 nucleotides of target sequence, and the term "substantially adjacent" is intended to refer to such situations.

It will also be appreciated from the foregoing that the second and third probes must hybridise to the arm region of the first probe such that the second and third probes are "adjacent", or substantially so, which terms are intended to have the same meanings as defined above. However, as the second and third probes together constitute one strand of the promoter, it is very much to be desired that they hybridise in an adjacent manner so as to provide optimum promoter activity: the inventors have found that the amount of promoter activity is greatly reduced even if a single nucleotide gap occurs between the second and third probes.

It will be apparent to those skilled in the art that the order of addition of probes in the formation of the nucleic acid complex is not critical: the third probe may, for example, be hybridised to the first probe before the second probe and sample are added. Alternatively, for example, all three probes may be simultaneously mixed with the sample containing the target molecule.

It will be further apparent to those skilled in the art that the second and third probes, which jointly provide one of the strands of the RNA polymerase promoter, are not covalently joined and the promoter sequence thus contains a "nick" in the phosphodiester backbone of one of the strands. For the sake of clarity, it is mentioned that the aforementioned first,

second and third probes are elsewhere described and referred to in this specification as "template", "complement" and "split complement" respectively.

Preferred promoters for use in the invention are those recognised by bacteriophage polymerases, especially those promoters recognised by one of T3, T7 or SP6 polymerase. These will generally comprise a minimum of 17 or 18 bases, essentially double-stranded. The sequence of the double-stranded T3 RNA polymerase promoter (described in the prior art) is:

i) 5' AATTAACCCTCACTAAA 3'
3' TTAATTGGGAGTGATTT 5'
or
ii) 5' TTA TTA ACC CTC ACT AAA 3'
3' AAT AAT TGG GAG TGA TTT 5'

i) = Seq ID No. 1

ii) = Seq ID No. 2

(A number of variant T3 promoter sequences are also known, especially those in which the first three bases of the non-template strand [the upper strand shown above] are 5' TTA 3', rather than AAT.)

The sequence of the T7 RNA polymerase promoter (described in the prior art) is:

5' TAATACGACTCACTATA 3'
3' ATTATGCTGAGTGATAT 5' (Seq ID No. 3)

The sequence of the SP6 RNA polymerase promoter (described in the prior art) is:

5' ATTTAGGTGACACTATA 3'
3' TAAATCCACTGTGATAT 5' (Seq ID No. 4)

One of the strands of the promoter is provided by the first probe. Typically this will be the "sense" (+) strand, which is transcribed by the polymerase. Accordingly the first

probe will generally comprise a stretch of "template" nucleic acid to be transcribed. The template will desirably comprise sequences which facilitate capture (e.g. hybridisation) of the resulting RNA transcript and/or detection. In addition, the first probe may desirably contain sequences (e.g. a "+12 sequence") adjacent to the promoter sequence, which serve to increase the activity of the promoter. Specific instances of such sequences are disclosed in the examples below. A "+12 sequence" is so-termed because it consists of 12 bases immediately downstream (i.e. at positions +1 to +12) of the promoter, and causes enhanced transcription levels.

The inventors have elucidated the optimum sequence of +12 regions for the T7 polymerase (discussed in greater detail below) - it is not known at present if these are also optimum for, say, T3 and SP6 polymerases. If, as is possible, SP6 and T3 polymerases have different optimum +12 regions, it would be a simple matter for the person skilled in the art to identify the relevant sequence by trial-and-error, with the benefit of the present disclosure.

The sequences of preferred +12 regions, for inclusion in the template portion of the first probe, (in respect of T7 polymerase) are shown below in Table 1. The most active +12 region (giving greatest transcription) is at the top, with the other sequences shown in decreasing order of preference.

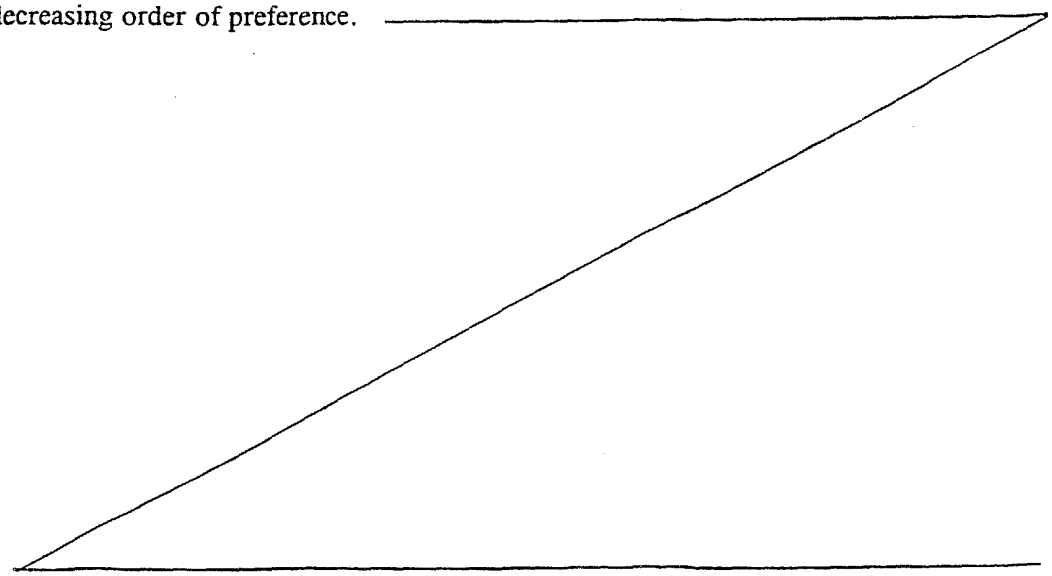


Table 1 Alternative template +1 to +12 sequences for T7 polymerase, in descending order of transcription efficiency (Seq ID Nos. 5-14).

5' ATCGTCAGTCCC 3'
5' GCTCTCTCTCCC 3'
5' ATCCTCTCTCCC 3'
5' GTTCTCTCTCCC 3'
5' GATGTGTCTCCC 3'
5' GTTGTGTCTCCC 3'
5' ATCCTCGTGCCC 3'
5' GCTCTCGTGCCC 3'
5' GTTCTCGTGCCC 3'
5' GTTGTGGTGCCC 3'

(The 5 base is numbered as +1, being the first base downstream from the end of the promoter sequence, the 3 base as +12).

In a further embodiment, the template portion of the complex (generally on the first probe) could contain sequences that can be used to isolate, identify, detect, quantify or amplify the *de novo* synthesised RNA transcripts (see, for example, WO 93/06240, US 5,554,516, or, for example, using molecular beacon sequences such as those disclosed by Tyagi & Kramer 1996 Nature Biotech 14, 303-308). These sequences are conveniently placed adjacent to, and downstream of, a +12 region (as described above) and may comprise, but are not limited to, one or more of the following: unique "molecular beacon" sequences; capture sequences; and detection probe complementary sequences.

In principle, the seventeen bases of the promoter sequence may be partitioned between the second and third probes in any manner, provided that, in combination, the second and third probes provide one strand of the full promoter sequence. In practice, the inventors have found that optimum results are generally obtained when the second probe provides 2 to 4 (preferably 3) bases at the 5' end of the promoter sequence, with the rest of the

promoter (15 to 13 bases) being contributed by the third probe. In addition, the inventors have found that promoter activity may be enhanced by including some additional bases (typically 1-3 bases or more) at the 3' end of the third probe (e.g. by providing at least some bases complementary to the +12 sequence on the template strand, so that the +12 sequence becomes at least partially double stranded).

Any of the first, second or third probes may comprise DNA, peptide nucleic acid (PNA), locked nucleic acid (LNA), (less preferably RNA) or any combination thereof. It will, however, generally be desirable that those portions of the probes which constitute the promoter comprise conventional DNA, so as to ensure recognition by the relevant polymerase. The terms "nucleic acid complex", "nucleic acid molecule" and "nucleic acid probe" should accordingly not be construed as being limited to complexes, molecules or probes (respectively) which consist solely of conventional nucleic acid, but also encompass complexes, molecules or probes which comprise non-conventional nucleic acid (such as PNA or LNA) or non-nucleic acid portions.

PNA is a synthetic nucleic acid analogue in which the sugar/phosphate backbone is replaced by a peptide-linked chain (typically of repeated N-(2-aminoethyl)-glycine units), to which the bases are joined by methylene carbonyl linkages. PNA/DNA hybrids have high Tm values compared to double stranded DNA molecules, since in DNA the highly negatively-charged phosphate backbone causes electrostatic repulsion between the respective strands, whilst the backbone of PNA is uncharged. Another characteristic of PNA is that a single base mis-match is, relatively speaking, more destabilizing than a single base mis-match in heteroduplex DNA. Accordingly, PNA is useful to include in probes for use in the present invention, as the resulting probes have greater specificity than probes consisting entirely of DNA. Synthesis and uses of PNA have been disclosed by, for example, Orum *et al*, (1993 Nucl. Acids Res. **21**, 5332); Egholm *et al*, (1992 J. Am. Chem. Soc. **114**, 1895); and Egholm *et al*, (1993 Nature **365**, 566).

LNA is a synthetic nucleic acid analogue, incorporating "internally bridged" nucleoside analogues. Synthesis of LNA, and properties thereof, have been described by a number of authors: Nielsen *et al*, (1997 J. Chem. Soc. Perkin Trans. **1**, 3423); Koshkin *et al*, (1998

Tetrahedron Letters 39, 4381); Singh & Wengel (1998 Chem. Commun. 1247); and Singh *et al*, (1998 Chem. Commun. 455). As with PNA, LNA exhibits greater thermal stability when paired with DNA, than do conventional DNA/DNA heteroduplexes. However, LNA can be synthesised on conventional nucleic acid synthesising machines, whereas PNA cannot.

In addition to non-conventional nucleic acids (such as LNA, PNA or nucleic acids containing base analogues), any one or more of the probes of use in the invention may comprise one or more destabilizing moieties.

The destabilizing moiety is a chemical entity which is generally unable to undergo base pairing and hydrogen bonding in the normal manner as usually occurs when complementary strands of nucleic acid become hybridised.

All manner of molecules may be suitable for use as a destabilizing moiety, although some compounds are specifically preferred, as described below. With the benefit of the present specification, the person skilled in the art will be able to test other compounds and readily select those which confer the appropriate degree of destabilization so as to prevent the hybridization of probes in the absence of target nucleic acid of interest. Particularly preferred, as a matter of convenience, are those compounds which are commercially available in a form (e.g. as phosphoramidites) which facilitates their incorporation into synthetic oligonucleotides using conventional automated solid phase nucleic acid synthesisers.

Destabilizing moieties which cannot base pair, but which nevertheless are capable of forming flexible folds and/or hairpin structures, are especially suitable. One such preferred destabilizing moiety comprises hexaethylene glycol (abbreviated herein as "Hex") (see Figure 5), which may be present singly or in tandem up to *n* times (where *n* can be any number 1, but conveniently has a maximum value of 5). In a particularly preferred embodiment, the third probe comprises one Hex molecule, where the number of bases opposite the destabilising moiety in the arm region of the first probe should be three to four bases. An alternative preferred destabilizing moiety comprises a plurality of

alkylene (especially methylene) repeats. Particularly preferred are penta- or hexa-methylene spacers.

Other, less preferred, destabilizing moieties may alternatively be used. These include, but are not limited to, inosine, Virazole™ (N[1]-[1- -D ribofuranosyl] 3-carboxamido-1,2,4,-triazole), Nebularin™ (N[9]-[1- -D ribofuranosyl]-purine), nitropyrrole, ribose, propyl or combinations of the above eg. propyl-Hex-propyl, propyl-Hex-Hex-propyl, etc. Propyl may be replaced by, for example, ethyl, butyl, pentyl, heptyl, octyl etc. The number of bases opposite the destabilizing moiety in the arm region of the reciprocal probe should be x, where x is an integer greater than or equal to 1. The exact number of bases will of course depend on the size of the destabilizing moiety and the value of n.

The following may be used as a guide: for each Hex molecule in the destabilizing moiety, the opposite probe should preferably comprise 3-4 bases (preferably 3) (i.e. X is between 3n and 4n); for each other molecule or radical mentioned above present in the destabilizing moiety, the opposite probe should preferably comprise a single base, with the exception of the following: butyl - two bases, pentyl - two bases, heptyl - three bases, and octyl - four bases.

The chemicals described above and used as destabilizing moieties are all commercially available (e.g. from Glen Research, USA).

The person skilled in the art will appreciate how to select appropriate conditions, materials and sequences for the probes, in order to ensure that the complex of the first, second and third probes (and hence formation of the functional RNA promoter) occurs in a target dependent manner. In essence, the degree of complementarity between the arm regions of the first and second probes must be such that, in the conditions employed, they will not hybridise unless stabilised by hybridisation of the respective foot regions of the first and second probes to the target.

Generally therefore, the foot regions of the first and second probes will comprise at least 10 bases, preferably at least 20 bases, and more preferably at least 25 bases. There is no

upper limit on the size of the foot regions (which may, for example, comprise several kilobases). However, in practice, the probes will normally be *in vitro* synthesised oligonucleotides and so it will be preferred for the foot regions to comprise no more than about 75 bases.

In contrast, the number of complementary bases between the arm regions of the first and second probes will normally be no more than 25, typically less than 15, and optimally between 5 and 13 bases, such that the arm regions will not (under the assay conditions employed) become hybridised to each other in the absence of target.

In preferred embodiments, the invention provides a method of generating a signal in a target-dependent manner (i.e. creation of the complex and hence formation of the functional promoter) and causing amplification of this signal (generation of multiple RNA transcripts under the control of the promoter) in a system which may require the use of a single enzyme type (RNA polymerase), without the need for additional enzymes (e.g. DNA polymerases) to bring about the amplification. This is significant as the reaction conditions for optimum activity of RNA and DNA polymerases are generally mutually exclusive.

Detection Methods

RNA produced in accordance with the invention could be detected in a number of ways, optionally following amplification (most preferably by means of an isothermal amplification step e.g. as disclosed in US 5,399,491 and US 5,480,784). For example, newly-synthesised RNA could be detected in a conventional manner (e.g. by gel electrophoresis), with or without incorporation of labelled bases during the synthesis.

Alternatively, for example, newly-synthesised RNA could be captured at a solid surface (e.g. on a bead, or in a microtitre plate), and the captured molecule detected by hybridisation with a labelled nucleic acid probe (e.g. radio-labelled, or more preferably labelled with an enzyme, chromophore, fluorophore and the like). Preferred enzyme labels include horseradish peroxidase and alkaline phosphatase.

One preferred detection method involves the use of molecular beacons or the techniques of fluorescence resonance energy transfer ("FRET"), delayed fluorescence energy transfer ("DEFRET") or homogeneous time-resolved fluorescence ("HTRF"). Molecular beacons are molecules which a fluorescence signal may or may not be generated, depending on the conformation of the molecule. Typically, one part of the molecule will comprise a fluorophore, and another part of the molecule will comprise a "quencher" to quench fluorescence from the fluorophore. Thus, when the conformation of the molecule is such that the fluorophore and quencher are in close proximity, the molecular beacon does not fluoresce, but when the fluorophore and the quencher are relatively widely-separated, the molecule does fluoresce. The molecular beacon conveniently comprises a nucleic acid molecule labelled with an appropriate fluorophore and quencher.

One manner in which the conformation of the molecular beacon can be altered is by hybridisation to a nucleic acid, for example inducing looping out of parts of the molecular beacon. Alternatively, the molecular beacon may initially be in a hair-pin type structure (stabilised by self-complementary base-pairing), which structure is altered by hybridisation, or by cleavage by an enzyme or ribozyme.

FRET (Fluorescence Resonance Energy Transfer) occurs when a fluorescent donor molecule transfers energy via a nonradiative dipole-dipole interaction to an acceptor molecule. Upon energy transfer, which depends on the R^{-6} distance between the donor and acceptor, the donor's lifetime and quantum yield are reduced and the acceptor fluorescence is increased or sensitised.

Another approach (DEFRET, Delayed Fluorescence Energy Transfer) has been to exploit the unique properties of certain metal ions (Lanthanides e.g. Europium) that can exhibit efficient long lived emission when raised to their excited states (excitation = 337 nm, emission = 620 nm). The advantage of such long lived emission is the ability to use time resolved (TR) techniques in which measurement of the emission is started after an initial pause, so allowing all the background fluorescence and light scattering to dissipate. Cy5 (Amersham Pharmacia) (excitation = 620 nm, emission = 665 nm) can be used as the DEFRET partner.

HTRF (see WO92/01224; US 5,534,622) occurs where a donor (e.g. Europium) is encapsulated in a protective cage (cryptate) and attached to the 5' end of an oligomer. The acceptor molecule that has been developed for this system is a protein fluorophore, called XL665. This molecule is linked to the 3' end of a second probe. This system has been developed by Packard.

Amplification and detection of RNA or other nucleic acid molecules are further described in our prior patent applications WO 99/37805 and WO 99/37806.

In another embodiment, the newly-synthesised RNA, before or after amplification, results in formation of a ribozyme, which can be detected by cleavage of a particular nucleic acid substrate sequence (e.g. cleavage of a fluorophore/quencher dual-labelled oligonucleotide).

In a third aspect the invention provides a complex comprising three nucleic acid molecules: a target nucleic acid sequence; a first probe; and a second probe; wherein the first probe comprises, in the 5' to 3' direction, a template portion transcribable by an RNA polymerase, a template strand of an RNA polymerase promoter, and a target complementary portion which is hybridised to at least a 3' end region of the target sequence; and wherein the second probe is hybridised to the first probe adjacent or substantially adjacent to the 3' end of the target sequence, the second probe comprising part of the non-template strand complementary to the template strand of the promoter present in the first probe, the remaining part of the non-template strand of the promoter sequence being present at the 3' end of the target sequence; the arrangement being such that formation of the complex creates a functional double stranded RNA polymerase promoter with a discontinuity in the non-template strand, between the second probe and the target sequence.

Those skilled in the art will appreciate that both the second probe and the target sequence are required to hybridise to the first probe, in order to form the double stranded functional promoter. Accordingly, RNA transcripts of the template portion of the first probe are indicative of the presence in a sample of the target sequence. Thus the complex of the

third aspect of the invention provides the basis for an assay for detecting the presence in a sample of a nucleic acid sequence of interest.

In a fourth aspect therefore, the invention provides a method of detecting in a sample the presence of a nucleic acid sequence of interest; the method comprising the steps of: contacting a first and second probe as defined above, with the sample, so as to form the complex of the third aspect of the invention wherein the target sequence is the sequence of interest or is formed as a result of the presence in the sample of the sequence of interest; and detecting directly or indirectly RNA transcripts of the template portion of the first probe.

The target sequence may be RNA or DNA, and may be a sequence of interest, or may be formed as a result of the presence in the sample of the sequence of interest (e.g. by PCR, or by one of the processes disclosed in one of WO 93/06240, WO 94/29481, EP 0851033 or EP 0552931). The RNA transcript is conveniently amplified and detected by means of the methods described elsewhere in this specification.

In the complex of the third aspect of the invention, and the method of the fourth aspect of the invention, the discontinuity in the non-template strand of the promoter may, in principle, occur at any position (i.e. the 3' end of the target sequence may contribute any number of bases to the promoter sequence). In practice, it is preferred that the target sequence contributes between 1 and 5 bases, more preferably 3 bases, to the promoter sequence, such that the resulting promoter has optimal activity. It is also highly preferred that the second probe hybridises to the first probe immediately adjacent to the target sequence, so that the discontinuity in the non-template strand of the promoter is as small as possible. Again, this optimises promoter activity when the complex is formed.

The invention will now be further described by way of illustrative example and with reference to the accompanying drawings, wherein:

Figures 1 and 3 are schematic representations of a nucleic acid complex in accordance with the first aspect of the invention;

Figures 2, 4 and 6-9 are bar charts showing amount of RNA produced (in femtomoles) from various nucleic acid complexes;

Figure 5 is a schematic representation of a preferred destabilizing moiety for use in some embodiments of the invention; and

Figure 10 is a schematic representation of a nucleic acid complex in accordance with the third aspect of the invention.

Example 1

This example demonstrated that T7 RNA polymerase and three oligonucleotide probes could be used to detect a specific target sequence. Probe 1 ("template") contained, sequentially, a 34 base foot region complementary to the target sequence; an 8 base overlap sequence; the 17 base template strand of the T7 promoter; a +12 sequence and a capture and probe sequence for detection. Probe 2 ("complement") comprised a 31 base foot region complementary to the target sequence; an 8 base overlap sequence and the first three bases of the complementary (or non-template) strand of the T7 promoter. Probe 3 ("split complement") contained the remaining 14 bases of the complementary (or non-template) strand of the 17 base T7 promoter sequence. The example is illustrated schematically in Figure 1.

Referring to Figure 1, a complex formed by a hybridisation reaction comprises four nucleic acid molecules: a 120 base target sequence 2, a first probe 4 ("template"), a second probe ("complement") 6, and a third probe ("split complement") 8. The orientation (5' to 3') of the four molecules 2, 4, 6 and 8 is indicated.

The first probe/template 4 comprises, in the 5' to 3' direction: a template portion 4a which facilitates isolation and detection of RNA transcripts; a +12 sequence 4b to enhance promoter activity; a promoter sequence 4c which consists of 17 bases of the T7 promoter; an overlap region 4d to hybridise to a complementary portion 6b of the second probe/complement 6; and a foot region 4e comprising 34 bases to hybridise to a complementary portion of the target 2.

The second probe/complement 6, comprises in the 5' to 3' direction: a 31 base foot region 6a to hybridise to the target; an 8 base overlap region 6b to hybridise to the complementary portion 4d of the first probe 4; and a partial promoter region 6c consisting of the first three bases (TAA) of one strand of the T7 promoter.

The third probe 8, comprises the remaining 14 bases of the T7 promoter strand.

In the presence of target 2 and probe molecules 4, 6 and 8, a complex is formed in which the foot regions of first probe 4 and second probe 6 hybridise to the target in an adjacent or substantially adjacent manner, which in turn allows the complementary overlap portions 4d and 6b to hybridise. Hybridisation of the third probe 8 to the promoter sequence 4c of the first probe thus creates a functional, double stranded T7 promoter, one of the strands of which (formed by second and third probes 6 and 8) is discontinuous.

1.1 **Preparation of oligonucleotides**

All oligonucleotide probes were synthesised by phosphoramidite chemistry using an Applied Biosystems 380A synthesiser according to the manufacturer's instructions. Biotinylation of oligonucleotide probes was achieved by incorporation of a biotin phosphoramidite. Oligonucleotides functionalised with alkaline phosphatase were prepared using the manufacturers proprietary method (Oswel). All oligonucleotides were HPLC purified using standard techniques.

1.2 **Synthesis of RNA off hybridised oligonucleotides**

Hybridisation was achieved in an assay mixture that contained 10 fmol of probe 1, 50 fmol of probe 2, 25 fmol of probe 3 and 1 fmol of probe 4 (target for CFTR gene), together with T7 RNA polymerase buffer (Promega) (Promega; 40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl at final concentration). The reaction volume was made up to 20 μl with RNase-free distilled water (allowing for later additions of enzymes and NTPs). Control reactions contained probes 1, 2 and 3 without target (probe 4). The mixture was heated to 90°C for 3 minutes to denature the nucleic acids, then cooled (by ramping at 0.1°C/second) to 37°C. T7 RNA polymerase (25 units) and 2 μl rNTP mix from Amersham Pharmacia Biotech (20 mM of each r NTP: adenosine 5' -triphosphate

(ATP), guanosine 5 -triphosphate (GTP), cytidine 5'-triphosphate (CTP) uridine 5'-triphosphate (UTP)) were added and the mixture incubated at 37°C for 3 hours.

1.3 Capture and detection of synthesised RNA

20 μl of assay sample was added to 145 μl hybridisation buffer (50 mM Tris-HCl, pH 8.0, 1 M NaCl, 20 mM EDTA and 0.1% BSA) containing 0.9 pmol biotinylated capture oligonucleotide (specific for the RNA to be detected) and 6 pmol specific alkaline phosphatase oligonucleotide in streptavidin coated wells. Incubation (60 minutes at room temperature, shaking at 300 rpm) allowed the RNA to be immobilised on the wells via the biotinylated capture probe and annealed to the detection probe. Unbound material was removed from the wells by washing four times with TBS/0.1% Tween-20, then once with alkaline phosphatase substrate buffer (Boehringer Mannheim). Finally, alkaline phosphatase substrate buffer containing 4-nitrophenyl phosphate (5mg/ml) was added to each well. The plate was incubated at 37°C in a Labsystems EIA plate reader and readings were taken at 405 nm every 2 minutes. Results are presented in Figure 2, which is a chart showing amount of RNA produced (in femtomoles), in the presence (+) or absence (-) of 1 fmol of target, for duplicate samples.

1.4 List of oligonucleotides

Probe 1 (template) Seq ID No. 15

5' TGCCTCCTTGTCTCCGTTCTGGATATCACCCGATGTGGCTCTCTCTCCCTA TAGTGAGTCGTATTAATTTCGAAGGTGTTTCCTATGATGAATATAGATACAGAA GCG 3' (phosphate blocked)

Probe 2 (complement) Seq ID No. 16

5' GCCTGGCACCATTAAAGAAAATATCATCTTTTTCGAAATTAA 3'

Probe 3 (split complement) Seq ID No. 17

5' TACGACTCACTATA 3'

Probe 4 (target) Seq ID No. 18

5'GTTGGCATGCTTTGATGACGCTTCTGTATCTATATTCATCATAGGAAACAC
CAAAGATGATATTTTCTTTAATGGTGCCAGGCATAATCCAGGAAAACTGAGAAC
AGAATGAAATTCTTC 3'

Sequence of transcribed RNA Seq ID No. 19

5' GGGAGAGAGAGCCACAUCGGGUGAUAUCCAGAACGGAGACAAGGAG
GCA 3'

Capture Probe Seq ID No. 20

5' TCTGCTGCCTGCTTGTCTGCGTTCT 3' (5' biotinylated)

Detection probe Seq ID No. 21

5' GGATATCACCCG 3' (3' alkaline phosphatase labelled)

Example 2

This example demonstrated that a Hexaethylene glycol linker (Hex) positioned 3 bases from the 3' end of Probe 3 (i.e. the split complement probe) increased the amount of signal obtained. The example is illustrated schematically in Figure 3. Corresponding integers are denoted using the same reference numerals adopted in Figure 1. H marks the approximate position of the hexaethylene residue incorporated in the split complement probe.

2.1 **Preparation of oligonucleotides**

All oligonucleotide probes were prepared and functionalised as described in Example 1.1. Hex incorporation was accomplished by reaction of the growing chain with 18-dimethoxytrityl hexaethylene glycol, 1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite. All oligonucleotides were HPLC purified using standard techniques.

The oligonucleotides used were identical to those described in Example 1, with the exception of the variant Probe 3 (split complement) oligo (referred to below as "Probe 5"), which contained a Hex between the 11th and 12th bases (counting from the 5' end).

2.2 **Synthesis of RNA off hybridised oligonucleotides**

Hybridisation was achieved in an assay mixture that contained 10 fmol of probe 1, 50 fmol of probe 2, 50 fmol of probe 3 or probe 5 (Hex containing variant of probe 3) and 1 fmol of probe 4 (target for CFTR gene), together with T7 RNA polymerase buffer (Promega). The reaction volume was made up to 20 μl with RNase-free distilled water (allowing for later additions of enzymes and NTPs). Control reactions contained probes 1, 2 and 3, or probes 1, 2 and 5, without target (probe 4). The mixture was heated to 90°C for 3 minutes to denature the nucleic acids, then cooled (by ramping at 0.1°C / second) to 37°C. T7 RNA polymerase (25 units) and 2 μl rNTP mix (Amersham Pharmacia Biotech) were added and the mixture incubated at 37°C for 3 hours.

2.3 **Capture and detection of synthesised RNA**

20 μl of assay sample was processed as described in Example 1.3. Results are presented in Figure 4, which is a chart showing amount of RNA produced in the presence (+) or absence (-) of target, for complexes without ("No Hex") or with a hexaethylene residue in the split complement probe.

Example 3

This example demonstrated that increasing the length of the split complement probe at the 3' end by 1, 2 or 3 bases increased the amount of RNA signal observed.

3.1 Preparation of oligonucleotides

All oligonucleotide probes were prepared and purified as described in the preceding examples.

3.2 Synthesis of RNA off hybridised oligonucleotides

Hybridisation was achieved in an assay mixture that contained 10 fmol of probe 1, 50 fmol of probe 2, 50 fmol of probe 3 (14 base split complement), 4 (15 base split complement), 5 (16 base split complement) or 6 (17 base split complement) and 1 fmol of probe 7 (target for CFTR gene), together with T7 RNA polymerase buffer (Promega). All reactions also contained 50 ng salmon sperm DNA (Sigma) and 5% Polyethylene glycol 300 (Sigma). The reaction volume was made up to 20 µl with RNase-free distilled water (allowing for later additions of enzymes and NTPs). Control reactions contained probes 1, 2 and one of probes 4, 5 or 6, without target (probe 7). The mixture was heated to 90°C for 3 minutes to denature the nucleic acids, then cooled (by ramping at 0.1°C / second) to 37°C. After 1 hr, T7 RNA polymerase (Promega) (25 units) and 2 µl rNTP mix (Amersham Pharmacia Biotech).

3.3 Capture and detection of synthesised RNA

20 µl of assay sample was processed as described in Example 1.3. Results are presented in Figure 6, which is a chart showing amount of RNA produced in reactions having a split complement probe of 14, 15, 16 or 17 bases in length.

3.4 List of oligonucleotides

Probe 1 (template) (Seq ID No. 22)

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGCTCTCTCTCCCTATAGTGAGT CGTATTAATTTCGAAGGTGTTTCCTATGATGAATATAGATACAGAAGCG 3'

(phosphate blocked)

Probe 2 (complement) – as Probe 2, Example 1

Probe 3 (14 base split complement) – as Probe 3, Example 1

Probe 4 (15 base split complement) – as Probe 3, Example 1 but with additional G at 3' end

Probe 5 (16 base split complement) – as Probe 3, Example 1 but with additional GG at 3'end

Probe 6 (17 base split complement) – as Probe 3, Example 1 but with additional GGG at 3' end

Probe 7 (target) – as Probe 4, Example 1

Sequence of transcribed RNA (Seq ID No. 23)

5' GGGAGAGAGAGCGCUGAGGCUUGAGAGGAGAGACCGGAAGACGA 3'

Capture Probe (Seq ID No. 24)

5' TCTGCTCGTCTTCCGGTCTCTCCTC 3' (5' biotinylated)

Detection probe (Seq ID No. 25)

5' TCAAGCCTCAGC 3' (3' alkaline phosphatase)

Example 4

This example demonstrated that a three base deletion in a target sequence could be detected. The foot region of the complement probe was 30 bases whilst the foot region of the template probe was either 14 bases or 30 bases. The three base deletion was located 7 bases from the junction point on the template probe foot side of the junction.

4.1 Preparation of oligonucleotides

All oligonucleotide probes were prepared and purified as described previously. Octanediol was incorporated by reaction of the growing chain with 8-dimethoxytrityl octanediol, 1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite.

4.2 Synthesis of RNA off hybridised oligonucleotides

Hybridisation was achieved in an assay mixture that contained 10 fmol of probe 1 (14 base template foot), 50 fmol of probe 3, 50 fmol of probe 4 and 1 fmol of probe 5 (wild type

target sequence) or 1 fmol of probe 6 (mutant target sequence); 10 fmol of probe 2 (30 base template foot), 50 fmol of probe 3, 50 fmol of probe 4 and 1 fmol of probe 5 (wild type target sequence) or 1 fmol of probe 6 (mutant target sequence). All reactions also contained 50 ng salmon sperm DNA (Sigma) and 5 % Polyethylene glycol 300 (Sigma). T7 RNA polymerase buffer (Promega) was added. The reaction volume was made up to 20 µl with RNase-free distilled water (allowing for later additions of enzymes and NTPs). Control reactions excluded the target (probe 5 or 6). The mixture was heated to 90°C for 3 minutes to denature the nucleic acids, then cooled (by ramping at 0.1°C / second) to 37°C. After 1 hr, T7 RNA polymerase (Promega) (25 units) and 2 µl rNTP mix (Amersham Pharmacia Biotech) were added and the mixture incubated at 37°C for 3 hours.

4.3 Capture and detection of synthesised RNA

20 µl of assay sample was processed as described previously. Results are presented in Figure 7. Figure 7 shows the amount of RNA-produced for reactions in which the template comprised a 14 or 30 base foot region respectively ("A" and "B"), for wild type (i) or mutant (ii) target sequences, or controls (iii) with no target. The results demonstrate that it was readily possible to discriminate between the wild type and mutant targets.

4.4 List of oligonucleotides

Probe 1 (template probe with a 14 base foot region) (Seq ID No. 26)

5' TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGCTCTCTCTCCCTATAGTG

AGTCGTATTAATTTCGAAQATATCATCTTTGGT 3' (phosphate blocked)

Q = Octanediol

Probe 2 (template probe with a 30 base foot region) (Seq ID No. 27)

5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGCTCTCTCTCCCTATAGTGAGT

CGTATTAATTTCGAAQATATCATCTTTGGTGTTTCCTATGATGAAT 3'

Q = Octanediol

Probe 3 (complement probe) (Seq ID No. 28)

5' GCCTGGCACCATTAAAGAAATTCGAAATTAA 3'

Probe 4 (split complement probe) – as Probe 4, Example 3

Probe 5 (wild type target sequence) – as Seq ID No. 18, Example 1

5' GTTGGCATGCTTTGATGACGCTTCTGTATCTATATTCATCATAGGAAACAC
CAAAGATGATATTTTCTTTAATGGTGCCAGGCATAATCCAGGAAAACTGAGAAC
AGAATGAAATTCTTC 3'

Bold text represents the 3 bases that are deleted in the mutant.

Probe 6 (mutant target sequence) (Seq ID No. 29)

5'GATGACGCTTCTGTATCTATATTCATCATAGGAAACACCAATGATATTTTCTT
TAATGGTGCCAGG CAT AAT CCA GG 3'

[Arrow represents the position of the 3 base deletion. The 3 base deletion is located 7 bases from the junction.]

Sequence of transcribed RNA (as Seq ID No. 23, Example 3)

Capture Probe (as Seq ID No. 24, Example 3)

Detection probe (as Seq ID No. 25, Example 3)

Example 5

This example demonstrated that 23S rRNA in total RNA purified from *Escherichia coli* K12 could be detected.

5.1 Preparation of oligonucleotides

All oligonucleotide probes were prepared and purified as described previously.

5.2 Preparation of total RNA

E. coli was grown in Luria-Bertani medium (10 g l^{-1} bacto-tryptone, 5 g l^{-1} yeast extract and 10 g l^{-1} sodium chloride) at 37 °C until the culture reached an OD_{600} of 1.0. A Qiagen RNeasy® Mini Kit was used to purify the total RNA. Cells were harvested and lysed according to the manufacturer's instructions. RNA was quantified using GeneQuant II (Amersham Pharmacia Biotech) according to the manufacturer's instructions and aliquots were stored at –80 °C until ready for use.

5.3 Synthesis of RNA off hybridised oligonucleotides

Hybridisation was achieved in an assay mixture that contained 1 fmol of probe 1, 5 fmol of probe 2, 5 fmol of probe 3 and 10, 1 or 0.1 ng of total RNA from *E. coli* K12. 50 ng salmon sperm DNA (Sigma) and 5 % Polyethylene glycol 300 (Sigma) were also included in all reactions. T7 RNA polymerase buffer (Promega) was added and the reaction volume made up to 20 µl with RNase-free distilled water (allowing for later additions of enzymes and NTPs). Control reactions contained probes 1, 2 and 3 without any target RNA. The mixture was heated to 95 °C for 5 minutes and then cooled (by ramping at 0.1°C / second) to 37 °C. After 1 hr, T7 RNA polymerase (Promega) (25 units) and 2 µl rNTP mix (Amersham Pharmacia Biotech) were added and the mixture incubated at 37 °C for 3 hours. The reactions were stored at –80 °C.

5.4 **Amplification of the RNA signal**

To further amplify the RNA transcribed from the T7 promoter in the complex, a linear DNA template (probe 4) was used, which contained a single stranded T7 promoter sequence. A 10 µl aliquot of each reaction was added to a mix containing 20 fmol of probe 4, 8 µl T7 RNA polymerase buffer, 50 µM dNTPs, 2 mM rNTPs, 51 Units T7 RNA polymerase and 4 Units *Bst* DNA polymerase. The volume was made up to 40 µl with RNase-free distilled water.

The mixture was incubated at 37°C for 3 hours. The RNA from the initial reaction (5.3) hybridised with probe 4 and was extended by the Bst polymerase, forming a fully functional double stranded RNA promoter, which then produced multiple RNA transcripts of the second DNA template, probe 4.

5.5 Capture and detection of synthesised RNA

40 µl of assay sample was processed as described previously. Results are presented in Figure 8. Figure 8 shows the amount of RNA produced in reactions using varying amounts (10, 1 or 0.1ng) of total RNA from *E. coli* K12 as target, compared with a control reaction (no target).

5.6 List of oligonucleotides

Probe 1 (template probe) (Seq ID No. 30)

5' TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCGCTCTCTCTCCCTATAGTG AGTCGTATTAATTTCGAAQGGCATGACAACCCGAACACCAGTGAT 3'

Q = Octanediol

Probe 2 (complement probe) (Seq ID No. 31)

5' GCATTTAGCTACCGGGCAGTGCCATTTTCGAAATTAA 3'

Probe 3 (split complement probe) – as Probe 4, Example 3

Probe 4 (2nd DNA template probe) - (Seq ID No. 32)

5' TGCCTGCTTGTCTGCGTTCTGGATATCACCCGAGTTCTCGCTTCCTATAGT GAGTCGTATTAATTTCTCGTCTTCCQGGTCTCTCCTCTCAAGCCTCAGCGCTCTC TCTCCC 3'

Q = Octanediol

Sequence of transcribed RNA to be detected (Seq ID No. 33)

5' GGAAGCGAGAACUCGGGUGAUAUCCAGAACGCAGACAAGCAGGCA 3'

Capture Probe - as Seq ID No. 20, Example 1

Detection probe - as Seq ID No. 21, Example 1

Example 6

This example demonstrated that a T3 promoter and T3 RNA polymerase could be used instead of a T7 promoter and T7 RNA polymerase. A 14 base split complement probe was used for the 17 base T3 promoter.

6.1 Preparation of oligonucleotides

All oligonucleotide probes were prepared and purified as described previously.

6.2 Synthesis of RNA off hybridised oligonucleotides

For the assay containing a T7 promoter, 10 fmol probe 1, 50 fmol probe 2, 50 fmol probe 3 and 1 fmol probe 4 were used. For the assay containing a T3 promoter, 10 fmol probe 5, 50 fmol probe 6, 50 fmol probe 7 and 1 fmol probe 4 were used. 50 ng salmon sperm DNA (Sigma) and 5% Polyethylene glycol 300 (Sigma) were also included in all reactions. T7 RNA polymerase buffer (Promega) was used with both T7 (Promega) and T3 RNA polymerases (Promega) and the reaction volume made up to 20 µl with RNase-free distilled water (allowing for later additions of enzymes and NTPs). Control reactions contained probes 1, 2 and 3 or probes 5, 6 and 7. The mixture was heated to 90°C for 3 minutes and then cooled (by ramping at 0.1°C/second) to 37°C. After 1 hr, T7 RNA polymerase (25 units) or T3 RNA polymerase (25 units) and 2 µl rNTP mix (Amersham Pharmacia Biotech) were added and the mixture incubated at 37°C for 3 hours. The reactions were stored at -80°C.

6.3 Capture and detection of synthesised RNA

20 µl of assay sample was processed as described previously. Results are presented in Figure 9.

Figure 9 shows the amount of RNA produced in reactions using T7 or T3 RNA polymerase. As expected, the reaction was specific in that T7 RNA polymerase would only produce RNA from a T7 promoter, not from a T3 promoter; and vice versa for T3 RNA polymerase. T3 polymerase produced slightly more RNA than T7 polymerase but, in this system, the background signal (in the absence of target) was much higher for T3.

List of oligonucleotides

Probe 1 (T7 template) (Seq ID No. 34)
5' TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCCTTCTCTCTTCCTATAGTG
AGTCGTATTAATTTCGAAGGTGTTTCCTATGATGAATATAGATACAGAAGCG 3'

Probe 2 (T7 complement) - as Seq ID No. 16, Example 1

Probe 3 (T7 split complement) - as Seq ID No. 17, Example 1

Probe 4 (target) (Seq ID No. 35)

5'CCTCCTCTAGTTGGCATGCTTTGATGACGCTTCTGTATCTATATTCATCAT
AGGAAACACCAAAGATGATATTTTCTTTAATGGTGCCAGGCATAATCCAGGAAA
ACTGAGAACAGAATGA 3'

Probe 5 (T3 template) (Seq ID No. 36)
5'TCGTCTTCCGGTCTCTCCTCTCAAGCCTCAGCCTTCTCTCTTCCTTTAG
TGAGGGTTAATTATTTCGAAGGTGTTTCCTATGATGAATATAGATACAGAAGCG
3'

Probe 6 (T3 complement) (Seq ID No. 37)

5' GCCTGGCACCATTAAAGAAAATATCATCTTTTTCGAAATAAT 3'

Probe 7 (T3 split complement) (Seq ID No. 38)

5' TAACCCTCACTAAA 3'

Sequence of transcribed RNA (Seq ID No. 39)

5' GGAA GAG AGA AGG CUG AGG CUU GAGAGGAGAGACCGGAAGACGA 3'

Capture Probe – as Seq ID No. 24, Example 3

Detection probe – as Seq ID No. 25, Example 3

Example 7

This example illustrates the complex of the third aspect of the invention, by reference to Figure 10.

Referring to Figure 10, a complex comprises a first probe 20, a second probe 22 and a target nucleic acid 24. The first probe 20 comprises a transcribable portion 20a, a template strand 20b of an RNA polymerase promoter (such as the T3, T7 or SP6 RNA promoter), and a target complementary portion 20c which is hybridised to 3' end of the target sequence 24.

The second probe 22 is hybridised to the first probe 20 adjacent to the target sequence 24. The second probe 22 comprises bases which are complementary to part (preferably the majority, e.g. 13-15 bases) of the template strand of the promoter on first probe 20 (i.e. the second probe 22 comprises part, preferably the majority, of the non-template strand of the promoter). The remainder of the non-template strand (typically 4-2 bases) is contributed by the 3' end of the target sequence 24. Accordingly, the complex is such that it comprises a functional double stranded RNA polymerase promoter which, in the

presence of a relevant RNA polymerase and ribonucleotide triphosphates, causes synthesis of RNA transcripts 26 of the template portion 20a of the first probe.

The RNA transcripts 26 may be detected, preferably following an optional amplification step, to indicate the presence of the target sequence 24, which may be the sequence of interest or which may have been generated in turn by the presence of the sequence of interest (e.g. by PCR, or by means of one of the other processes described in the prior art, such as WO 93/06240, WO 94/29481 or EP 0851033).

Claims

1. A complex formed by a hybridisation reaction comprising four nucleic acid molecules; the complex comprising a target nucleic acid molecule and first, second and third nucleic acid probe molecules; wherein the first probe comprises a foot region which is complementary to a first portion of the target and is hybridised thereto, and an arm region which is substantially non-complementary to the target; the second probe comprises a foot region which is complementary to a second portion of the target, such that the foot region of the second probe is hybridised to the target adjacent or substantially adjacent to the foot region of the first probe, the second probe also comprising an arm region which is substantially non-complementary to the target but which is complementary and hybridised to the arm region of the first probe; the third probe being complementary, at least in part, to a portion of the arm region of the first probe, such that the third probe is hybridised to the arm region of the first probe adjacent or substantially adjacent to the second probe; and wherein formation of the complex creates a functional double-stranded RNA polymerase promoter, one strand of the promoter being provided by the first probe, and the other strand being provided jointly by the second probe and by the third probe.

2. A complex according to claim 1, wherein at least one of the first, second or third probes comprises PNA and/or LNA.

3. A complex according to claim 2, wherein the first and/or second probe comprises PNA and/or LNA.

4. A complex according to any one of claims 1, 2 or 3, comprising a functional double stranded T3, T7 or SP6 RNA polymerase promoter.

5. A complex according to any one of the preceding claims, comprising single or double stranded sequence adjacent to the promoter which increases the activity of the promoter.

6. A complex according to claim 5, wherein one of said probes comprises a +12 sequence.

7. A complex according to claim 5, wherein the first probe comprises a +12 sequence.

8. A complex according to any one of the preceding claims, comprising a sequence which, when transcribed into RNA, facilitates isolation, identification, detection, quantification or amplification of the transcript.

9. A complex according to any one of the preceding claims, wherein one of said probes comprises a destabilizing moiety.

10. A complex according to any one of the preceding claims, wherein the second and third probes form a discontinuous sequence of an RNA polymerase promoter template strand.

11. A complex according to any one of claims 1-9, wherein the second and third probes form a discontinuous sequence of an RNA polymerase promoter non-template strand.

12. A method of detecting the presence of a target nucleic acid molecule in a sample, the method comprising the steps of: contacting the sample comprising the target with first and second nucleic acid probes, each probe comprising a foot region complementary to respective first and second portions of the target, which portions are adjacent or substantially so; wherein the first and second probes each further comprise an arm region substantially non-complementary to the target, at least part of the arm region of

the first probe being complementary to at least part of the arm region of the second probe, such that respective foot regions of the first and second probes hybridise to the target, allowing hybridisation of the complementary parts of the arm regions of the first and second probes; and causing to be present a third nucleic acid probe molecule which is complementary to a portion of the arm region of the first probe, such that the third probe hybridises to the first probe adjacent or substantially adjacent to the arm region of the second probe, thereby creating a functional double-stranded RNA polymerase promoter, one strand of the promoter being provided by the first probe, the other strand being provided jointly by the second and third probes; causing RNA synthesis from the RNA promoter; and detecting, directly or indirectly, the RNA so synthesised.

13. A method according to claim 12, performance of which results in the formation of a complex in accordance with any one of claims 1-11.

14. A method according to claim 12 or 13, wherein RNA produced from the functional RNA promoter is amplified prior to detection.

15. A method according to any one of claims 12, 13 or 14, wherein RNA produced from the functional RNA promoter is detected directly or indirectly via a method which involves use of a molecular beacon or fluorophore.

A complex comprising three nucleic acid molecules: a target nucleic acid sequence; a first probe; and a second probe; wherein the first probe comprises, in the 5' to 3' direction, a template portion transcribable by an RNA polymerase, and a template strand of an RNA polymerase promoter, a target complementary portion which is hybridised to at least a 3' end region of the target sequence; and wherein the second probe is hybridised to the first probe adjacent or substantially adjacent to the 3' end of the target sequence, the second probe comprising part of the non-template strand complementary to the template strand of the promoter present in the first probe, the remaining part of the non-template strand of the promoter sequence being present at the 3' end of the target sequence; the arrangement being such that formation of the complex creates a functional double stranded RNA polymerase promoter, with a discontinuity in the non-template strand, between the second probe and the target sequence.

16. A method of detecting in a sample the presence of a nucleic acid target sequence; the method comprising the steps of: contacting a first and second probe as defined above, with the sample, so as to form the complex of claim 16; and detecting directly or indirectly RNA transcripts of the template portion of the first probe.